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Degeneration of the nigrostriatal dopamine system is linked to the pathophysiology of Parkinson's disease. Similarly, the psychostimulant drug, methamphetamine also produces relatively selective damage to nigrostriatal dopamine neurons and is rapidly becoming a widespread problem and drug of abuse throughout the U.S. However, the neurochemical underpinnings that mediate methamphetamine toxicity and Parkinson's disease have escaped definition.

We propose that several variables common to methamphetamine toxicity and Parkinson's disease, each of which may be important but alone are insufficient, account for the neurodegeneration of the nigrostriatal dopamine path. It is hypothesized that the convergence of excitotoxicity, free radicals and a depleted bioenergetic state produces damage to dopamine neurons. Moreover, environmental stressors, which also increase free radicals, excitatory amino acids, and alter energy metabolism, predispose dopamine neurons to damage. Consequently, environmental stress may be synergistic with oxidative and metabolic insults as well as glutamate to culminate in dopamine cell death. ***The major objective is to examine in rats the interaction between environmental stress and methamphetamine and the convergent action of excitotoxicity, bioenergetic stress, and oxidative stress to produce damage to nigrostriatal dopamine neurons.*** A multidisciplinary approach of *in vivo* and *in vitro* biochemical and histochemical methods will be used.

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Bryan K. Yamamoto 7/10/00

PI Signature Date

Table of Contents

INTRODUCTION

Degeneration of the nigrostriatal dopamine system is linked to the pathophysiology of Parkinson's disease. Similarly, the psychostimulant drug, methamphetamine, also produces relatively selective damage to nigrostriatal dopamine neurons and is rapidly becoming a widespread problem and drug of abuse throughout the U.S. However, the neurochemical underpinnings that mediate methamphetamine toxicity and Parkinson's disease have escaped definition.

We propose that several variables common to methamphetamine toxicity and Parkinson's disease, each of which may be important but alone are insufficient, account for the neurodegeneration of the nigrostriatal dopamine path. It is hypothesized that the convergence of excitotoxicity, free radicals and a depleted bioenergetic state produces damage to dopamine neurons. Moreover, environmental stressors, which also increase free radicals, excitatory amino acids, and alter energy metabolism, predispose dopamine neurons to damage. Consequently, environmental stress may be synergistic with oxidative and metabolic insults as well as glutamate to culminate in dopamine cell death. *The major objective is to examine in rats the interaction between environmental stress and methamphetamine and the convergent action of excitotoxicity, bioenergetic stress, and oxidative stress to produce damage to nigrostriatal dopamine neurons.* A multidisciplinary approach of *in vivo* and *in vitro* biochemical and histochemical methods will be used. In addition, pharmacological strategies that we posit to be neuroprotective against methamphetamine, excitotoxicity, and bioenergetic and oxidative stress will be examined.

ANNUAL PROGRESS REPORT

We have made significant progress on both of the objectives described in the Statement of Work.

OBJECTIVE 1: To develop a chronic unpredictable stress and heat stress models and to use these paradigms to study the interaction between stress, methamphetamine, and excitotoxicity to dopamine neurons.

Results

Chronic Stress

The unpredictable stress paradigm is patterned after that described by Ortiz et al., 1996. Stressed and control subjects were weighed daily to monitor their health during the procedure. The procedure we have used is described below:

Day 1: 12 pm, cage rotation for 50 min; 1 pm, swim stress for 4 min.
Day 2: 11 am, cold room (4°) for 60 min; 6 pm, lights on, overnight.
Day 3: 12 pm, lights off for 3 hrs; 3 pm, cold isolation (4°) for 15 min.
Day 4: 6 pm, cage rotation for 50 min, 6 pm food/water deprivation overnight.
Day 5: 1 pm, swim stress for 3 min; 7 pm isolation housing overnight.
Day 6: 11 am, restraint stress for 60 min; 3 pm, lights off for 2 hours.
Day 7: 10 am, swim stress for 4 min; 4 pm, restraint stress for 60 min.
Day 8: 7 pm, lights on and food/water deprivation overnight.

Day 9: 10 am, cage rotation for 20 min; 7 pm, lights on overnight.
Day 10: 7 pm, isolation housing and food/water deprivation overnight.

Chronic stress exposed rats had significantly lower body weights throughout the 10 day period (Table 1). When injected with methamphetamine or MDMA, stressed rats had a higher mortality rate than non-stressed controls (Table 2).

Table 1. Effect of repeated stress on weight gain in Sprague-Dawley rats. Weight gain (grams) over 10 day period.

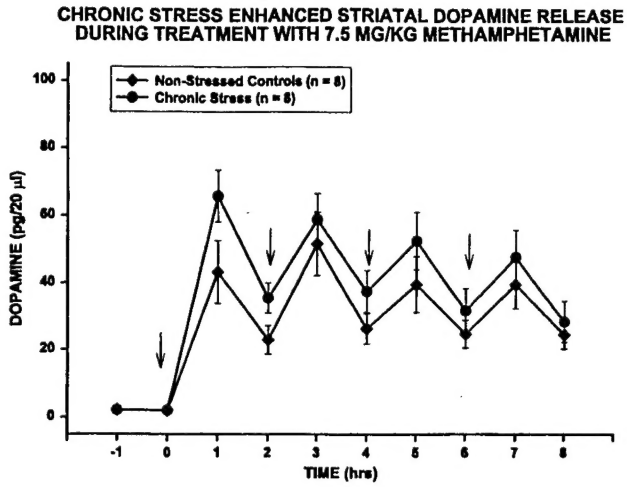
	NON-STRESSED CONTROLS	CHRONIC STRESS
METHAMPHETAMINE		
10 mg/kg	102.7 ± 4.0	35.9 ± 4.7 *
7.5 mg/kg	64.5 ± 2.7	42.9 ± 6.8 *
MDMA		
10 mg/kg	109.8 ± 4.3	35.5 ± 7.7 *

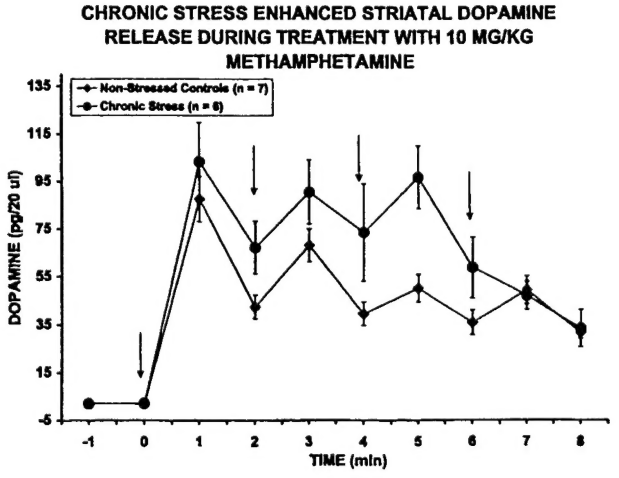
Table 2. Effect of drug treatment on mortality. Percentage of rats that died during drug treatment.

	NON-STRESSED CONTROLS	CHRONIC STRESS
METHAMPHETAMINE		
10 mg/kg	0 %	67 % *
7.5 mg/kg	0 %	0 %
MDMA		
10 mg/kg	25 %	0 %

At both the 7.5 and 10 mg/kg doses of methamphetamine, stressed rats exhibited more striatal dopamine release (Fig. 1), had greater hyperthermic responses to methamphetamine (Fig. 2), and more marked long-term depletions of dopamine in striatal tissue when examined 7 days after the drug administration (Fig. 3).

Figure 1





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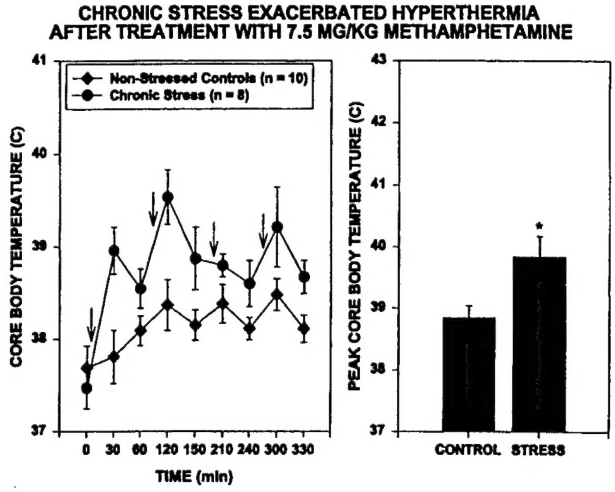

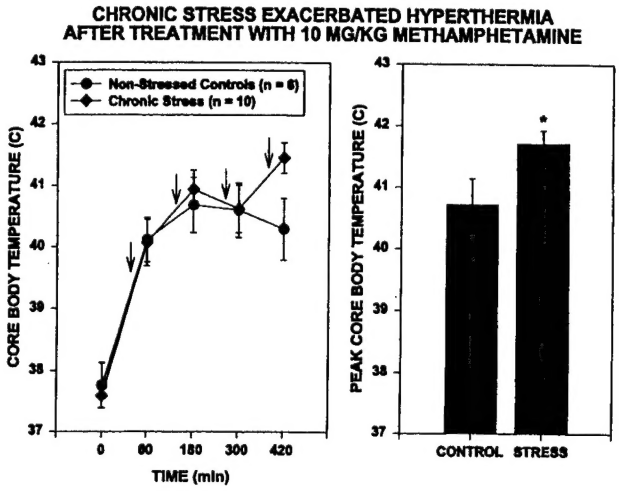

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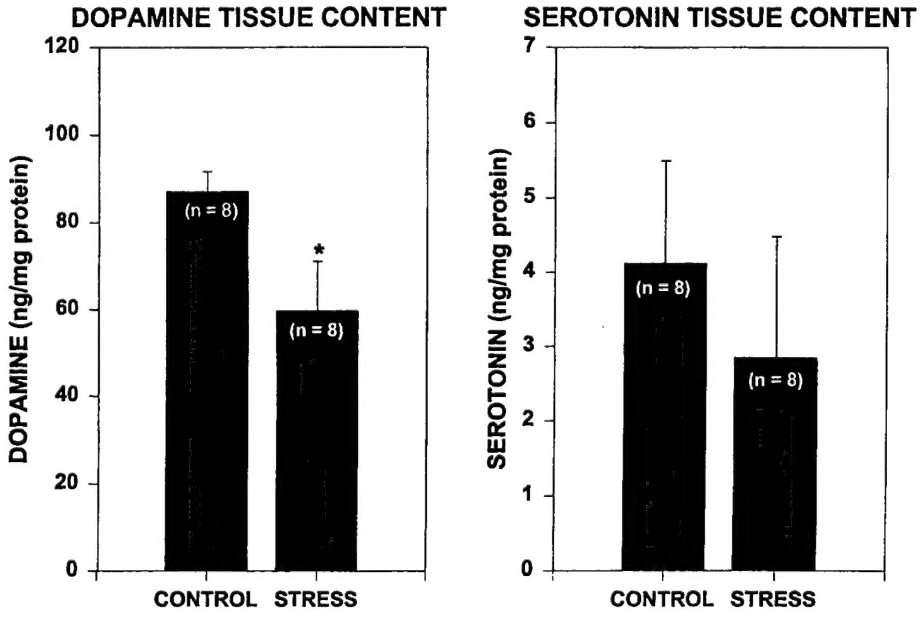
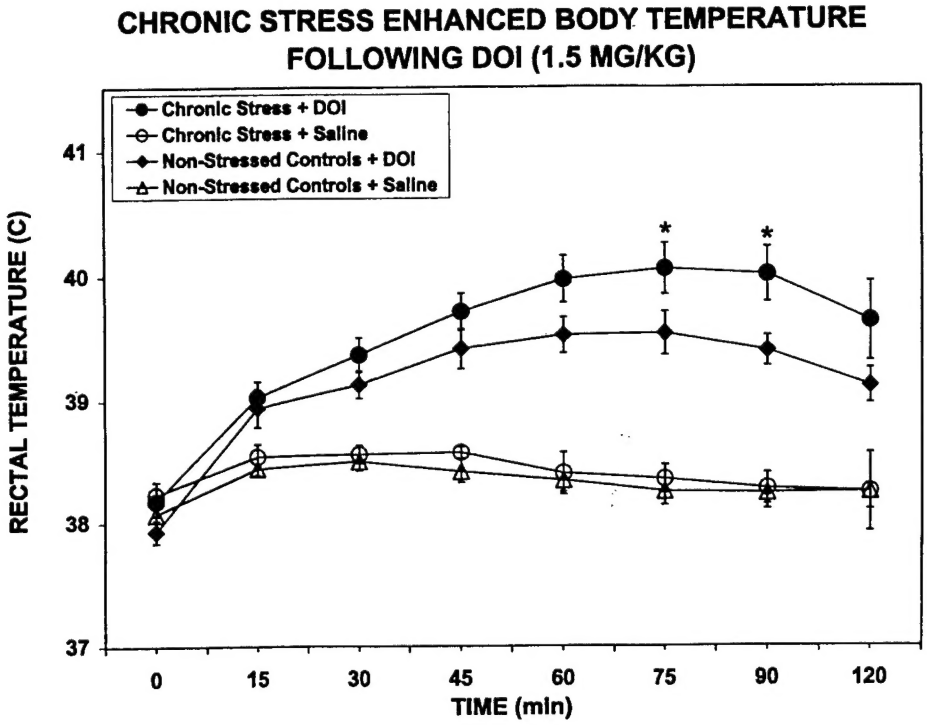


To understand the possible mechanisms underlying the exaggerated hyperthermic responses to methamphetamine, separate groups of stressed and unstressed control rats were challenged with the 5HT2A/C agonist, DOI and body temperatures monitored. The results indicate that stressed rats exhibit a significantly greater hyperthermic response to DOI (Fig. 4).

Figure 4



In summary, the results of these experiments indicate that prior exposure to stress enhances the vulnerability of the striatum to the acute and long-term neurotoxic consequences of methamphetamine and that this effect is the result of a 5HT-2 mediated hyperthermia.

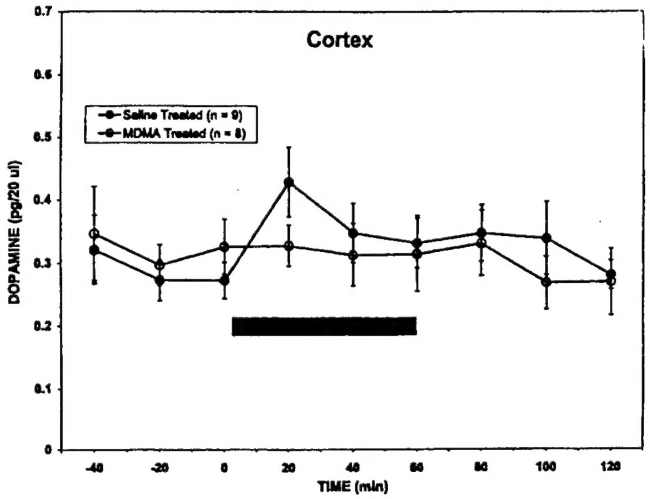
Acute Stress

In contrast to the above studies that have focussed on the effects of chronic stress on the response to challenge administrations of amphetamines, no studies to data have examined the effects of prior exposure to neurotoxic doses of amphetamines on the subsequent reactivity to an acute stressor such as restraint stress. It was hypothesized that prior exposure to neurotoxic doses of the methamphetamine analogue, MDMA (Ecstasy), would alter the normal neurotransmitter responses to an acute restraint stress episode. Rats were injected with either saline or MDMA and one week later, exposed to a restraint stress for one hour while simultaneously monitoring dopamine, serotonin (5HT) and/or glutamate release in the cortex and hippocampus.

Results

The results show that prior treatment with MDMA blocks the normal glutamate, dopamine and 5HT increases in hippocampus and/or cortex during a restraint stress episode but not the plasma corticosterone increase. More specifically, in saline treated rats, dopamine in the prefrontal cortex (Fig. 5) and glutamate in the cortex (Fig. 6) and hippocampus (Fig. 7) increased in stressed rats. 5HT in the prefrontal cortex of saline treated rats also increased

(Fig. 8) in a pattern similar to that of dopamine whereas 5HT in the hippocampus showed a delayed rise in saline treated rats (Fig. 9). MDMA treated rats showed no transmitter release in the dorsal hippocampus or the prefrontal cortex in response to restraint stress (Figs. 5-9). The hatched bars indicate the period of restraint stress.



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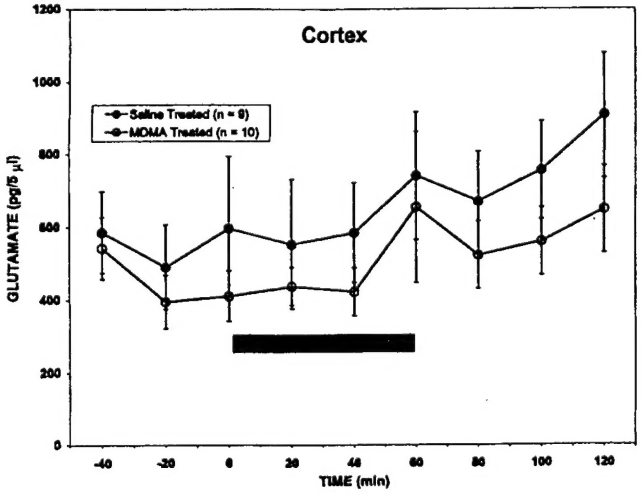

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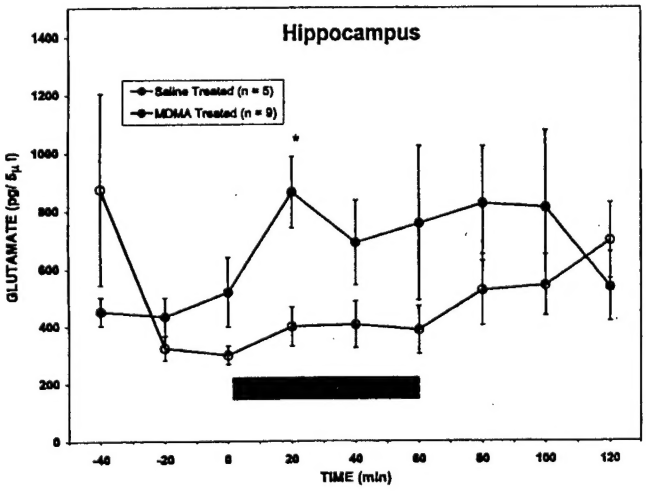

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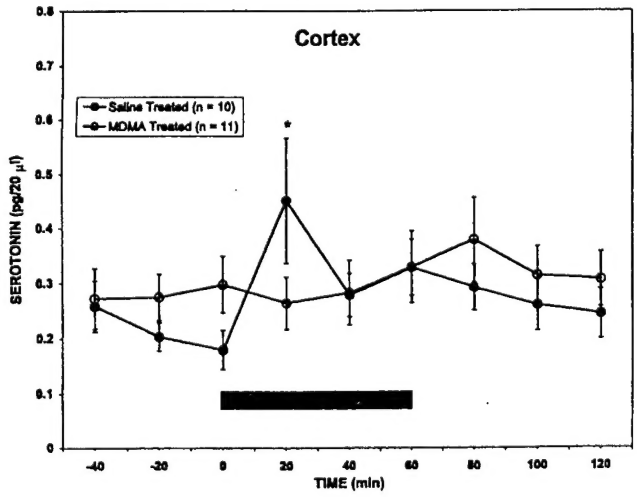

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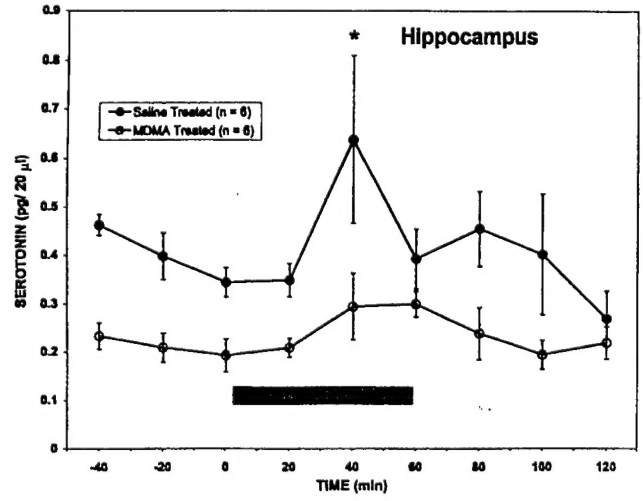


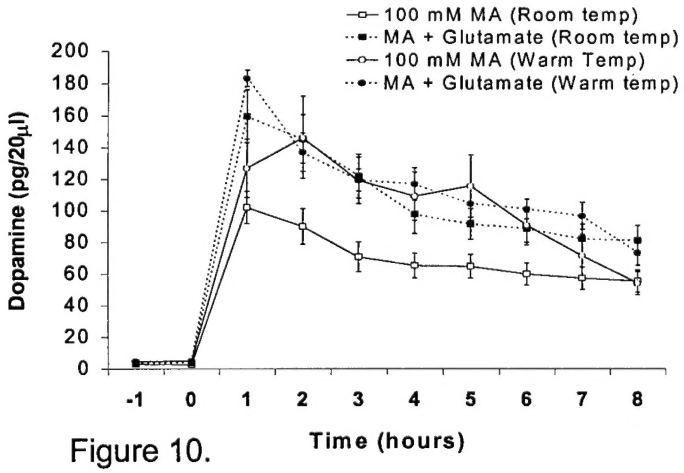
In conclusion, depletions of forebrain 5HT by MDMA attenuate the stress-induced release of neurotransmitters in the cortex and hippocampus. These findings suggest that the integrity of the 5HT system is important in regulating normal biochemical responses to stress. This is the first evidence of an increase in 5HT release in the frontal cortex produced by stress. Moreover, the results suggest that both 5HT and dopamine in the cortex are initial responses to a stressor and contrast the delayed 5HT response observed in the hippocampus.

Heat Stress and Glutamate

Hyperthermia, glutamate and dopamine are known to mediate, in part, the neurotoxicity to methamphetamine. The objective was to determine if local MA perfusion, in combination with a local rise in glutamate and/or an increase in body temperature, was sufficient to produce neurotoxicity. By isolating the contributing factors (dopamine, glutamate, hyperthermia), it was possible to examine the relative contribution of each of these factors in mediating methamphetamine toxicity. Systemic injections of methamphetamine induce hyperthermia (2-5°C change) and an increase in both extracellular and glutamate in the striatum. Although local perfusion of methamphetamine releases dopamine to a similar degree, intrastriatal infusions of methamphetamine do not induce hyperthermia, do not increase extracellular levels of glutamate, and do not result in a loss of dopamine or 5HT content.

Results

The direct local perfusion of methamphetamine increased dopamine release similar to the effects of systemic methamphetamine (Fig. 10). Increased ambient temperatures (28-38°C) resulted in a sustained hyperthermic response that approximated the effects of systemic methamphetamine administration (peak rectal temperature 41.5 ± 0.09°C). Hyperthermia alone did not result in a depletion of monoamines, but in combination with methamphetamine resulted in enhanced dopamine release and a 30% depletion of dopamine tissue content 7 days later (Figs. 10 and 11). Perfusion of glutamate in combination with methamphetamine also enhanced striatal dopamine release compared to methamphetamine alone at either temperature tested (Fig. 10), but did not appear to exacerbate the depletion of dopamine tissue content (Fig. 11). However, glutamate perfusion with hyperthermia (in the absence of methamphetamine) did decrease 5HT tissue content (Fig. 12). Local perfusion of vehicle, glutamate (100 μM) at either temperature did not alter monoamine content.



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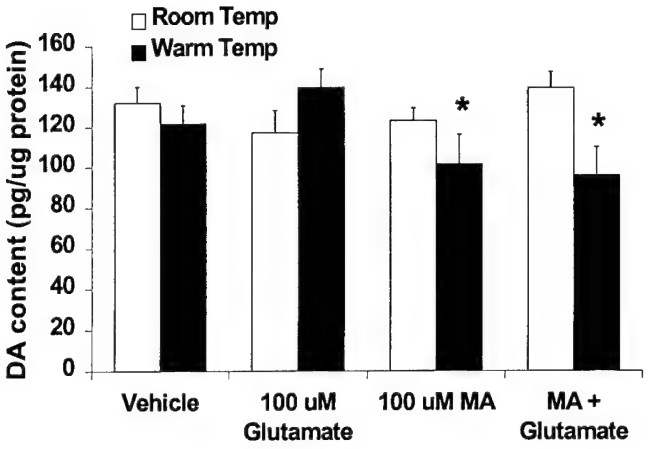

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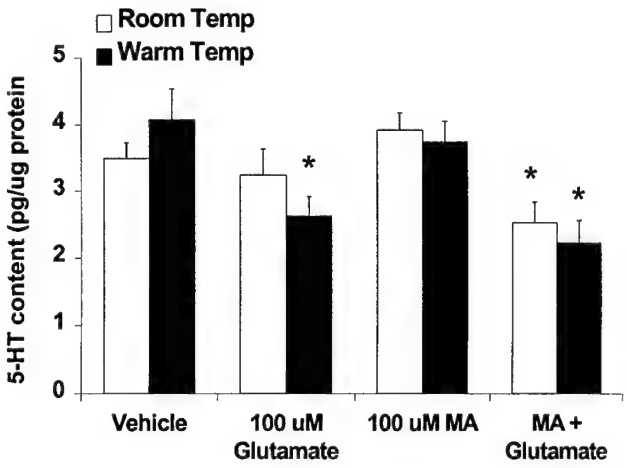


Figure 12.

OBJECTIVE 2 – To examine the interaction between methamphetamine toxicity and energy metabolism

Cytochrome Oxidase

The primary goal of these studies was to identify and characterize mitochondrial dysfunction following methamphetamine or MDMA administration. Specifically, the activity of complex IV (cytochrome oxidase) was determined following administration of high doses of methamphetamine or MDMA to determine if these psychostimulants had rapid or lasting effects on mitochondrial enzyme function.

Results

There was a rapid decrease in cytochrome oxidase (Cox) staining in the striatum (23-29%), nucleus accumbens (29-30%) and substantia nigra (31-43%), 2 h following administration of either MA and MDMA. This decrease in COx activity was transient and returned to control levels within 24 h.

Since the MA and MDMA-induced decrease in COx activity was localized to dopamine-rich regions, increased extracellular concentrations of dopamine may contribute to the inhibition of metabolic function via its metabolism to form quinones or other reactive oxygen species. These results support previous studies demonstrating that psychostimulants induce a rapid and transient decrease in striatal ATP stores and provide further evidence that these drugs of abuse can disrupt mitochondrial function. These findings were published (Burrows et al., Eur. Journal of Pharmacology 398: 11-18, 2000) and a reprint is appended.

Metabolic Inhibition and Methamphetamine

These studies examined, *in vivo,* the effect of local intrastriatal perfusion of methamphetamine on dopamine and glutamate release in relation to long-term changes in striatal neurotransmitter content. Interactions between inhibition of energy metabolism and direct perfusion of methamphetamine on the long-term decreases in dopamine and 5-HT content were also investigated.

Results

Methamphetamine (100 μM), the succinate dehydrogenase (Complex II) inhibitor malonate, or the combination of methamphetamine and malonate, was reverse-dialyzed into the striatum for 8 hours. The continuous local perfusion of methamphetamine alone increased dopamine release by 30 fold, similar to that seen following systemic administration, but did not increase glutamate or body temperature, and did not deplete neurotransmitter content. Malonate perfusion increased both dopamine and glutamate overflow, and dose dependently decreased dopamine content. 5-HT content was not as affected by malonate perfusions (200 mM malonate depleted dopamine by 66% and 5-HT by 40%). When methamphetamine was co-perfused with 200 mM malonate, dopamine content was reduced by 80% and to a greater extent compared with malonate alone. Co-perfusion of methamphetamine and 200 mM malonate did not enhance 5-HT loss.

Overall, the direct local perfusion of methamphetamine alone, which only increased extracellular dopamine in the absence of glutamate release and hyperthermia, was not toxic to striatal dopamine terminals but synergized with the local inhibition of energy metabolism to deplete dopamine content. Moreover, the inhibition of energy metabolism and the synergy with methamphetamine was more toxic to dopamine compared to 5-HT terminals. These findings were published (Burrows et al., *Journal of Pharmacology and Experimental Therapeutics* 292: 853-860, 2000) and a reprint is appended.

Metabolic Inhibition and MDMA Neurotoxicity

The acute and long-term effects of the local perfusion of 3,4-methylenedioxymethamphetamine and the interaction with the mitochondrial inhibitor malonate were examined in the striatum and compared to a similar perfusion of methamphetamine. 3,4-methylenedioxymethamphetamine (MDMA), methamphetamine, malonate, or the combination of malonate with 3,4-methylenedioxymethampetamine or methamphetamine was reverse dialyzed into the striatum for 8 hours via a microdialysis probe while extracellular dopamine and serotonin were measured. One week later, tissue immediately surrounding the probe was assayed for dopamine and serotonin tissue content.

Results

The local perfusion of MDMA and methamphetamine increased dopamine and serotonin release but did not produce long-term depletions of dopamine or serotonin in tissue. Malonate also increased both dopamine and serotonin release but, in contrast to the amphetamines, only produced long-term depletions in dopamine. The combined perfusion of MDMA /malonate or methamphetamine/malonate synergistically increased the release of dopamine and serotonin and produced a long-term depletion of dopamine in tissue. Depletions of serotonin concentrations in tissue were only observed following perfusion with MDMA /malonate.

These results support the conclusion that dopamine, compared to serotonin, neurons are more susceptible to mitochondrial inhibition. Moreover, malonate interacts with the toxic effects of MDMA and methamphetamine to damage dopamine neurons. The effects of MDMA in combination with malonate on serotonin neurons suggest a role for bio-energetic stress in 3,4-methylenedioxymethamphetamine-induced toxicity to serotonin neurons. Overall, these results highlight the importance of energy balance to the function of dopamine and serotonin neurons and to the toxic effects of MDMA and methamphetamine. A preprint of a manuscript submitted for publication is appended (Nixdorf et al., *Neuroscience*, submitted).

Other Studies

Role of Oxidative Stress and Methamphetamine Neurotoxicity

As methamphetamine-induced neurotoxicity has been proposed to involve oxidative stress, the levels of endogenous antioxidants glutathione, vitamin E and ascobate were determined after methamphetamine. Reduced and oxidized glutathione (GSH and GSSG, respectively), vitamin E, and ascorbate were measured in the striata of rats killed 2 or 24 h after a neurotoxic regimen of methamphetamine.

Results

At 2 h, methamphetamine increased GSH and GSSG (32.5% and 43.7%) compared to controls at 2 h. No difference was seen in glutathione at 24 h and in vitamin E and ascorbate at either time point. These findings indicate a selective effect of methamphetamine for the glutathione system and a role for methamphetamine in inducing oxidative stress. These findings will be published (Harold et al., *Eur. Journal of Pharmacology*, in press) and a proof of the article is appended.

Brain Derived Neurotrophic Factor and Methamphetamine Toxicity

Because brain derived neurotrophic factor (BDNF) provides neurotrophic support, regulates neurotransmitter activity including dopamine and 5HT, protects neurons from excitotoxic insults, and is decreased by stress, it was hypothesized that amphetamines alone and in combination with stress will alter BDNF protein levels. Therefore BDNF was measured in the hippocampus, cortex and striatum after MDMA alone or in combination with chronic stress.

Results

MDMA increased BDNF in the striatum (Fig. 13) and cortex (Fig. 14) of chronic stress rats 24 hours after drug injection. In contrast, MDMA decreased BDNF in the hippocampus of chronic stressed rats 24 hours after injection (Fig. 15). These changes only appeared in chronic stressed rats treated with MDMA. No significant changes were observed with MDMA in non-stressed rats.

Figure 13

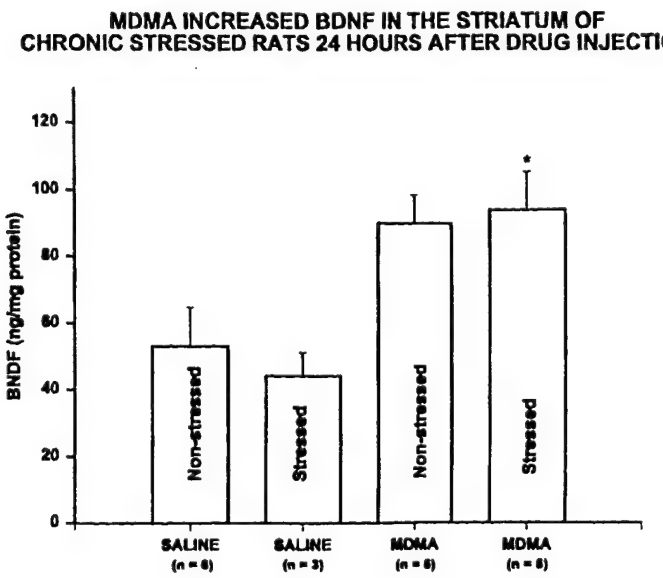
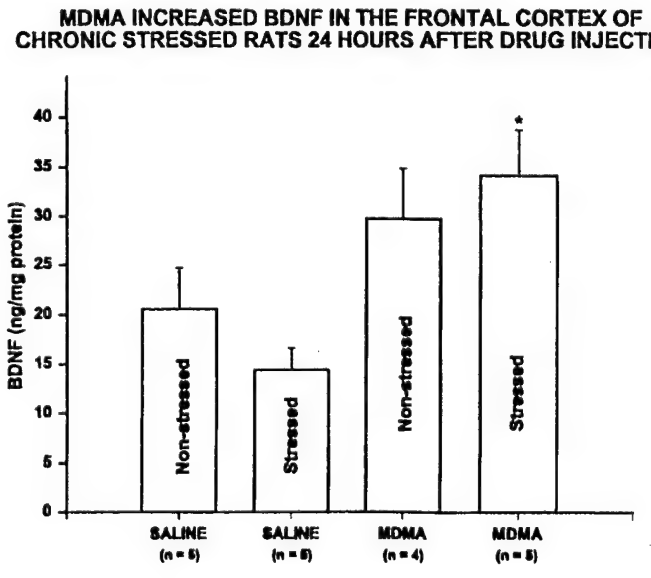


Figure 14



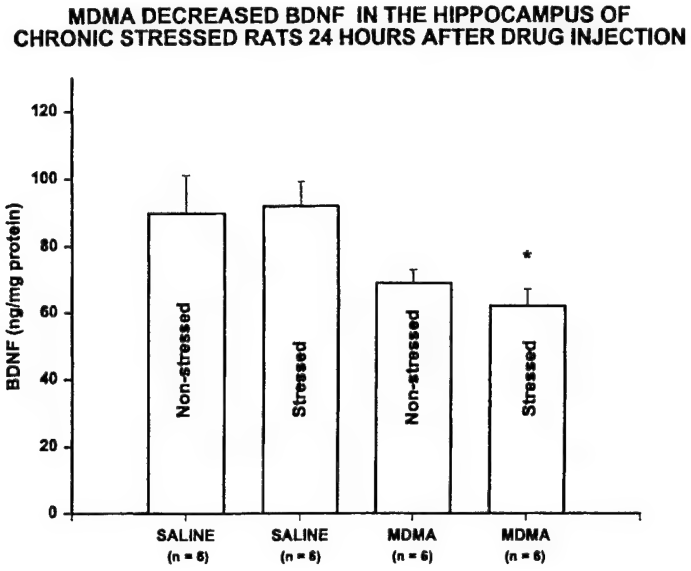


Figure 15

These studies provide evidence that BDNF protein concentrations are affected by MDMA following chronic stress in a manner that is brain region-dependent. The decrease in BDNF in the hippocampus of chronic stressed rats after MDMA may underlie the enhanced vulnerability of this brain region (vs. the striatum and cortex) to stress and MDMA toxicity.

KEY RESEARCH ACCOMPLISHMENTS

- Chronic unpredictable stress enhances the neurotoxicity of methamphetamine
- The enhanced neurotoxicity of methamphetamine subsequent to the exposure to chronic stress is mediated in part, through hyperthermia and a 5HT-2 receptor dependent mechanism.
- Neurotoxic doses of MDMA disrupt the normal pattern of transmitter release to a stressor and suggest that the integrity of the 5HT system is important in the regulation of the normal biochemical responses to stress.
- Hyperthermia synergizes with the pharmacological actions of methamphetamine to produce long-term depletions of dopamine in the striatum
- Mitochondrial function is inhibited by the amphetamines and synergizes with the acute pharmacological actions of these drugs to produce long-term depletions of striatal dopamine and 5HT.
- Striatal dopamine neurons are more vulnerable than 5HT neurons to the toxic effects of mitochondrial inhibition
- Methamphetamine produces oxidative stress
- MDMA synergizes with chronic stress to alter BDNF protein content in a brain region-dependent manner

REPORTABLE OUTCOMES (1999-2000)

Published Papers

Burrows, K., Nixdorf, W., and Yamamoto, B.K. Central administration of methamphetamine synergizes with metabolic inhibition to deplete striatal monoamines. Journal of Pharm. and Exptl. Therapeutics, 292: 853-860, 2000

Burrows, K.B., Gudelsky, G.A. and Yamamoto, B.K. Role of Metabolic Inhibition in Methamphetamine and MDMA Toxicity: Evidence for Decreased Mitochondrial Function following Drug Administration. European Journal of Pharmacology 398: 11-18, 2000.

Yamamoto, B.K. Roles for Metabolic and Oxidative Stress in Amphetamine Neurotoxicity. Neurotoxicology and Teratology (Conference report), in press.

Harold, C., Wallace, T., Friedman, R., Gudelsky, G.A., and Yamamoto, B.K. Methamphetamine selectively alters brain antioxidants. European Journal of Pharmacology, in press.

Submitted Papers

Nixdorf, W.L., Burrows, K.B., Gudelsky, G.A., and Yamamoto, B.K. Acute and long-term effects of 3,4-methylenedioxymethamphetamine on serotonin and dopamine: Interactions with inhibition of energy metabolism and comparisons to methamphetamine. Submitted

Matuszewich, L. Filon, M.E. Finn, D.A. Gudelsky, G.A. and Yamamoto, B.K. Altered forebrain neurotransmitter responses to immobilization stress following 5-HT depletions with MDMA. Submitted.

Abstracts and Presentations

Filon, M.E.., Matuszewich, L., Gudelsky, G.A., and Yamamoto, B.K. Long-term effects of MDMA on stress-induced transmitter release. Society for Neuroscience, 1999.

Nixdorf, W.L., Burrows, K.B., Gudelsky, G.A., and Yamamoto, B.K. Acute and long-term effects of intrastriatal perfusions of methamphetamine or MDMA. Society for Neuroscience, 1999.

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CONCLUSIONS

Environmental stress interacts with the metabolic and oxidative insults produced by methamphetamine to cause long-term depletions of brain dopamine and serotonin. These effects may be mediated by the 5HT2 receptor and alterations in brain derived neurotrophic factor. Moreover, prior exposure to the amphetamines disrupts the normal neurochemical responses to stress. It can be speculated that environmental stress may be a determinant in enhanced vulnerability of dopamine neurons to these toxic insults. Acute or repeated exposures to single or multiple stressors (e.g. heat stress, psychological stress or physical stress) can exacerbate the damaging effects of known or potential neurotoxins and heighten the neurotoxic potential of dopamine neurons to the comcomitant exposure to environmental stress and

and psychostimulants. These studies have important implications for drug abuse, neural mechanisms of stress, and the pathophysiology of Parkinson's disease.

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List of Appendices

1. Burrows, K.B., Gudelsky, G.A. and Yamamoto, B.K. Role of Metabolic Inhibition in Methamphetamine and MDMA Toxicity: Evidence for Decreased Mitochondrial Function following Drug Administration. European Journal of Pharmacology 398: 11-18, 2000.

2. Burrows, K., Nixdorf, W., and Yamamoto, B.K. Central administration of methamphetamine synergizes with metabolic inhibition to deplete striatal monoamines. Journal of Pharm. and Exptl. Therapeutics, 292: 853-860, 2000

3. Nixdorf, W.L., Burrows, K.B., Gudelsky, G.A., and Yamamoto, B.K. Acute and long-term effects of 3,4-methylenedioxymethamphetamine on serotonin and dopamine: Interactions with inhibition of energy metabolism and comparisons to methamphetamine. Submitted

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5. Abstracts

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Rapid and transient inhibition of mitochondrial function following methamphetamine or 3,4-methylenedioxymethamphetamine administration

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Abstract

Metabolic mapping of discrete brain regions using cytochrome oxidase histochemistry was used as a marker for alterations in mitochondrial function and cytochrome oxidase enzymatic activity in response to high doses of amphetamine derivatives. The activity of cytochrome oxidase, complex IV of the electron transport chain, was determined at three different time-points following administration of high doses of methamphetamine or 3,4-methylenedioxymethamphetamine (MDMA) (four injections of 10–15 mg/kg administered over 8 h). There was a rapid decrease in cytochrome oxidase staining in the striatum (23–29%), nucleus accumbens (29–30%) and substantia nigra (31–43%), 2 h following administration of either methamphetamine and MDMA. This decrease in cytochrome oxidase activity was transient and returned to control levels within 24 h. Since the methamphetamine and MDMA-induced decrease in cytochrome oxidase activity was localized to dopamine-rich regions, increased extracellular concentrations of dopamine may contribute to the inhibition of metabolic function via its metabolism to form quinones or other reactive oxygen species. These results support previous studies demonstrating that psychostimulants induce a rapid and transient decrease in striatal ATP stores and provide further evidence that these drugs of abuse can disrupt mitochondrial function. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Neurotoxicity; Cytochrome oxidase; Amphetamine; Histochemistry; Energy metabolism

1. Introduction

Increased metabolic stress compromises bioenergetic processes and has been hypothesized to contribute to lasting changes in the dopamine and serotonin (5-hydroxytryptamine, 5-HT) systems following high-dose methamphetamine administration. Evidence of metabolic stress following methamphetamine includes increased extracellular concentrations of lactate (Stephans et al., 1998) and a decrease in striatal ATP concentrations (Chan et al., 1994). Poblete and Azmitia (1995) have reported that 3,4-methylenedioxymethamphetamine (MDMA) increases the breakdown of glycogen in vitro, indicating that similar changes in metabolic function occur following administration of other substituted amphetamines. Acute administration of methamphetamine or its parent compound amphetamine, has been shown to rapidly (within 1 h) increase local cerebral glucose utilization in multiple brain regions (Pontieri et al., 1990; Porrino et al., 1984). In addition, high-dose treatment with methamphetamine or MDMA results in decreased cerebral glucose metabolism weeks to months following drug administration, suggesting lasting impairments in metabolic systems (Huang et al., 1999; McBean et al., 1990; Sharkey et al., 1991). Although these studies indicate that energy metabolism is altered following methamphetamine or MDMA administration, no studies have demonstrated directly that mitochondrial function itself is acutely or chronically compromised following high doses of psychostimulants.

Metabolic mapping using cytochrome oxidase histochemistry can be used to compare relative levels of enzy-

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matic activity in discrete brain regions (Hevner et al., 1995). The density of cytochrome oxidase staining is highly correlated with cytochrome oxidase activity as measured in tissue homogenates, but the histochemical technique has the advantage of higher anatomic resolution compared with biochemical measurements in tissue homogenates (Hevner et al., 1995). Unlike mapping using 2-deoxyglucose, which measures rapid changes in glucose utilization, cytochrome oxidase histochemistry can be used to measure changes in energy usage over a period of hours to weeks (Wong-Riley, 1989). The degree of cytochrome oxidase staining is believed to reflect the overall functional activity of neurons in that intense staining is associated with areas that have both a high level of excitatory input and high tonic firing rates (Kageyama and Wong-Riley, 1982; Mjaatvedt and Wong-Riley, 1991).

The primary goal of this study was to identify and characterize mitochondrial dysfunction following methamphetamine or MDMA administration. Specifically, the activity of complex IV (cytochrome oxidase) was determined following administration of high doses of methamphetamine or MDMA to determine if these psychostimulants had rapid or lasting effects on mitochondrial enzyme function. Based on the suggestion that psychostimulants increase the formation of nitric oxide (Abekawa et al., 1996; Zheng and Laverty, 1998), a known complex IV inhibitor (Cleeter et al., 1994; Lizasoain et al., 1996), and the finding of depleted energy stores following methamphetamine administration (Chan et al., 1994), it was hypothesized that high-dose methamphetamine or MDMA would decrease cytochrome oxidase histochemical staining in a brain region specific manner correlating with the ability of these drugs to increase extracellular concentrations of dopamine and 5-HT. Cytochrome oxidase activity was examined 2 h, 24 h, and 7 days following administration of the last dose of methamphetamine or MDMA. These time-points were chosen, in part, based on a previous study demonstrating a loss of striatal ATP at 1.5 h, but not 24 h following the same course of methamphetamine treatment (Chan et al., 1994). In addition, several studies have demonstrated that substituted amphetamines result in a rapid depletion of neurotransmitter content, followed by a transitory recovery (24 h post drug), and a lasting monoamine loss (generally measured 3 or 7 days post drug). Therefore, these time-points were also chosen to determine if changes in cytochrome oxidase activity correlate temporally with the known time-course of methamphetamine- and MDMA-induced monoamine loss.

2. Materials and methods

2.1. Subjects and drug administration

Male Sprague–Dawley rats ($N = 61$, 200–275 g) were housed, two to three animals per cage, in clear plastic shoe boxes. Animals were maintained on a 12-h light/dark cycle (lights on at 6:00 AM) with food and water available ad libitum throughout the experiments. All animal experiments were carried out in accordance with the National Institute of Health guide for the care and use of laboratory animals (NIH Publications No. 80-23). For all experiments, methamphetamine (10 mg/kg), MDMA (15 mg/kg), or an equivalent volume of vehicle (saline) was administered i.p. every 2 h for a total of four injections. Four animals died following administration of MDMA ($n = 2$) or methamphetamine ($n = 2$).

The protocol used in the study required the transcardial perfusion of rats with fixative, a technique that is not compatible with the measurement of dopamine and 5-HT tissue content. However, hyperthermia following psychostimulant administration has been shown to be a reliable predictor of toxicity (Bowyer et al., 1994; Che et al., 1995; Craig and Kupferberg, 1972). Therefore, rectal temperatures (taken every 30–60 min for 8 h following the first injection) were determined by the use of an RET-2 copper–constantan thermocouple rectal probe (Physitemp Instruments; Clifton, NJ) and a TH-8 thermalert thermometer (Sensortek; Clifton, NJ).

2.2. Cytochrome c oxidase histochemistry

Rats were killed 2 h ($n = 5$ per group), 24 h ($n = 7–10$ per group), or 7 days ($n = 5–6$ per group), following the last injection of drug or vehicle. Subjects were anesthetized with chloral hydrate and perfused transcardially with fixative (0.3% glutaraldehyde, 4% paraformaldehyde, and 2% sucrose in 0.1 M phosphate buffer, pH 7.4). Whole brains were post-fixed for 1 h, rinsed in 0.1 M phosphate buffer with 4% sucrose and cryoprotected by submersion in buffer with increasing concentrations of sucrose. Brains were sliced (40 μm) on a cryostat (−20°C) and rinsed on a shaker at 4°C overnight in phosphate buffer with 4% sucrose. To ensure that differences in cytochrome oxidase staining were not due to variations across staining runs, slices from comparable brain regions for the three drug treatment groups (saline, methamphetamine, MDMA) were placed in individual Netwell™ tissue processing wells (Electron Microscopy Sciences, Fort Washington, PA) and processed together for cytochrome oxidase activity according to a modification of the method by Wong-Riley (1979). Slices were then incubated (38°C) in phosphate-buffered saline (4% sucrose) with 3,3′-diaminobenzidine and cytochrome *c* for 3 h. Following repeated washing in buffer, slices were mounted on gel-coated slides and coverslipped for later analysis.

2.3. Data analysis

Images were captured from slides using a CCD camera (Sierra Scientific, Sunnyvale CA) and a Northern Light

box (Model B90, Imaging Research, St. Catherines, Ontario, Canada). The relative optical density (ROD score) of staining, a semi-quantitative measure of cytochrome oxidase activity, in various brain regions was determined using an MCID image analysis system (Imaging Research). Each area of interest was outlined in its entirety and the average pixel density determined for that nucleus. Approximately 4–12 densitometry measurements were made for each brain region within an animal. These comprised at least the left and right sides of two consecutive slices assayed in duplicate trays. For larger nuclei, additional consecutive slices were analyzed. The number of slices analyzed (one to three pairs) depended upon the size of the region examined (i.e., more slices were taken for regions with greater anterior/posterior length such as the caudate). This was to insure that staining for cytochrome oxidase activity did not show regional variability within nuclei examined (i.e., the anterior vs. posterior striatum). Variability between these measurements was very low. Although occasional hemispheric differences were noted, no systematic differences between hemispheres or across nuclei were found. Therefore, the densitometry measurements were averaged to give a single ROD score for each brain region within a given rat.

The density of background staining in white matter (corpus callosum) was subtracted from the total ROD score obtained in gray matter regions to give a measure of specific staining of cytochrome oxidase activity. Corrected ROD scores for tissue taken from animals killed at a given time-point were compared by two-way mixed factor analysis of variance (ANOVA) with drug treatment as the between-subjects factor and brain region as the within-subjects factor. Significant interactions were further analyzed by Newman–Keuls' test. Peak core temperature during drug administration was analyzed by one-way ANOVA followed by Newman–Keuls' test.

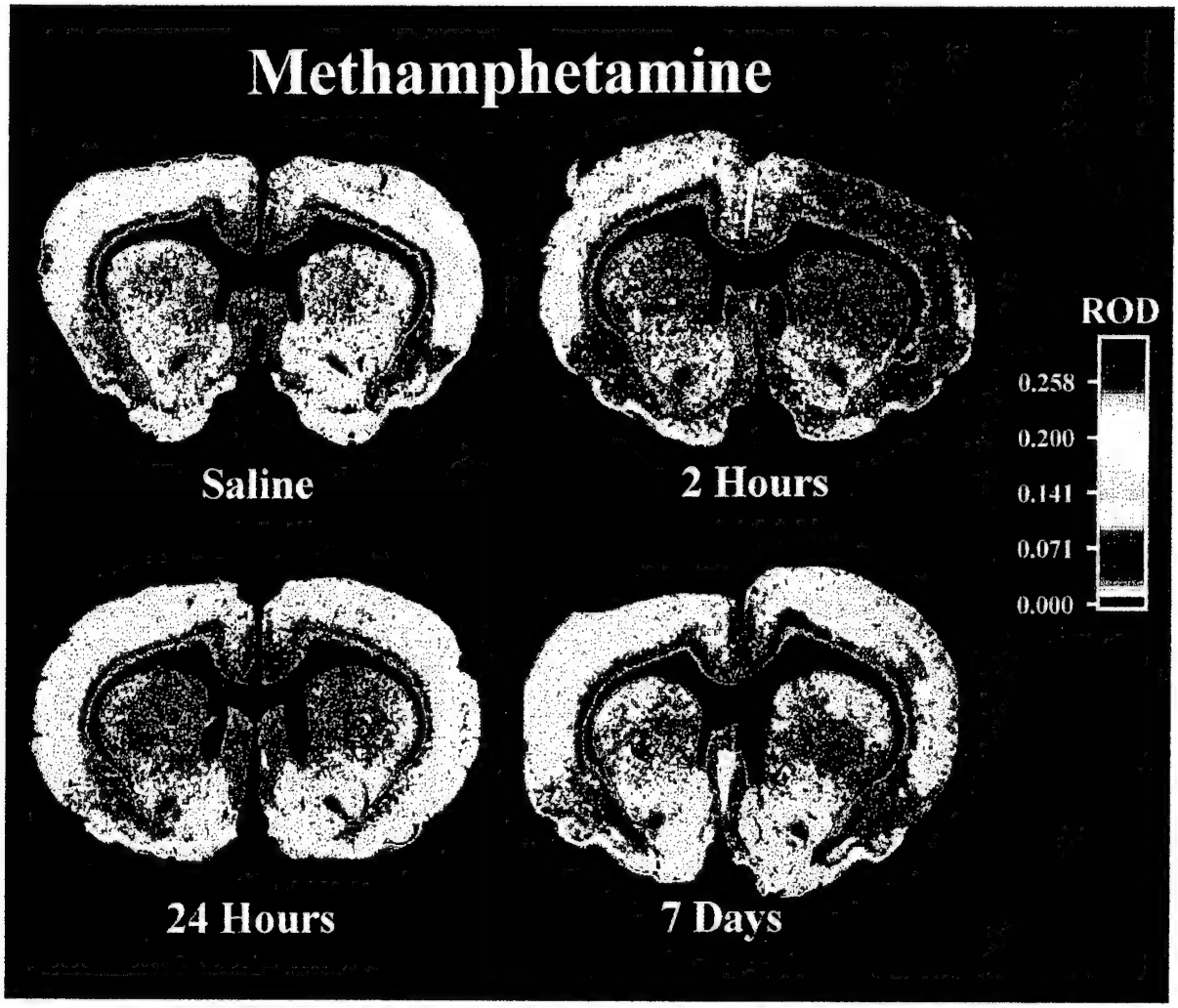

Fig. 1. Cytochrome oxidase histochemistry in representative coronal slices from animals treated with saline (top left) or methamphetamine (10 mg/kg) and killed 2 h (top right), 24 h (bottom left), or 7 days (bottom right) following the last dose of drug. Note the decrease in density of staining within the caudate and nucleus accumbens 2 h following methamphetamine administration (panel B) compared to a saline control rat (panel A).

2.4. Materials

The following drugs and chemicals were purchased from Sigma (St. Louis, MO): D-methamphetamine, MDMA, 3,3′-diaminobenzadine, paraformaldehyde, and cytochrome *c* (derived from horse heart). Glutaraldehyde (50% solution, biological grade) was obtained from Electron Microscopy Sciences. Doses of methamphetamine and MDMA are expressed as the salt. Sodium phosphate (monobasic and dibasic) was obtained from Fisher Scientific (Fair Lawn, NJ).

3. Results

The density of cytochrome oxidase staining varied significantly across brain region with the greatest staining in the nucleus accumbens and the lowest staining in the entopeduncular nucleus (main effect of region $F(9,108) = 71.5$, $P < 0.01$). There was a rapid decrease in cytochrome oxidase staining in the striatum (23–29%), substantia nigra (31–43%), and in both the core and shell subregions of the nucleus accumbens (29–30%) 2 h following administration of the last dose of either methamphetamine (Fig. 1) or MDMA (Fig. 2) (main effect of drug: $F(2,12) = 6.5$, $P < 0.01$; interaction: $F(18,108) = 2.4$, $P < 0.01$) (Fig. 3a). This decrease in cytochrome oxidase activity was transient and reversed to control levels within 24 h (Fig. 3b) and remained at basal levels 7 days post drug (Fig. 3c). Although there was a general trend towards a decrease in activity in most regions, cytochrome oxidase histochemistry was not significantly altered at any time-point in the other six regions examined (hippocampus, entopeduncular nucleus, motor thalamus, subthalamic nucleus, motor cortex, prefrontal cortex). In preliminary studies, no clear differences between the substantia nigra pars compacta and

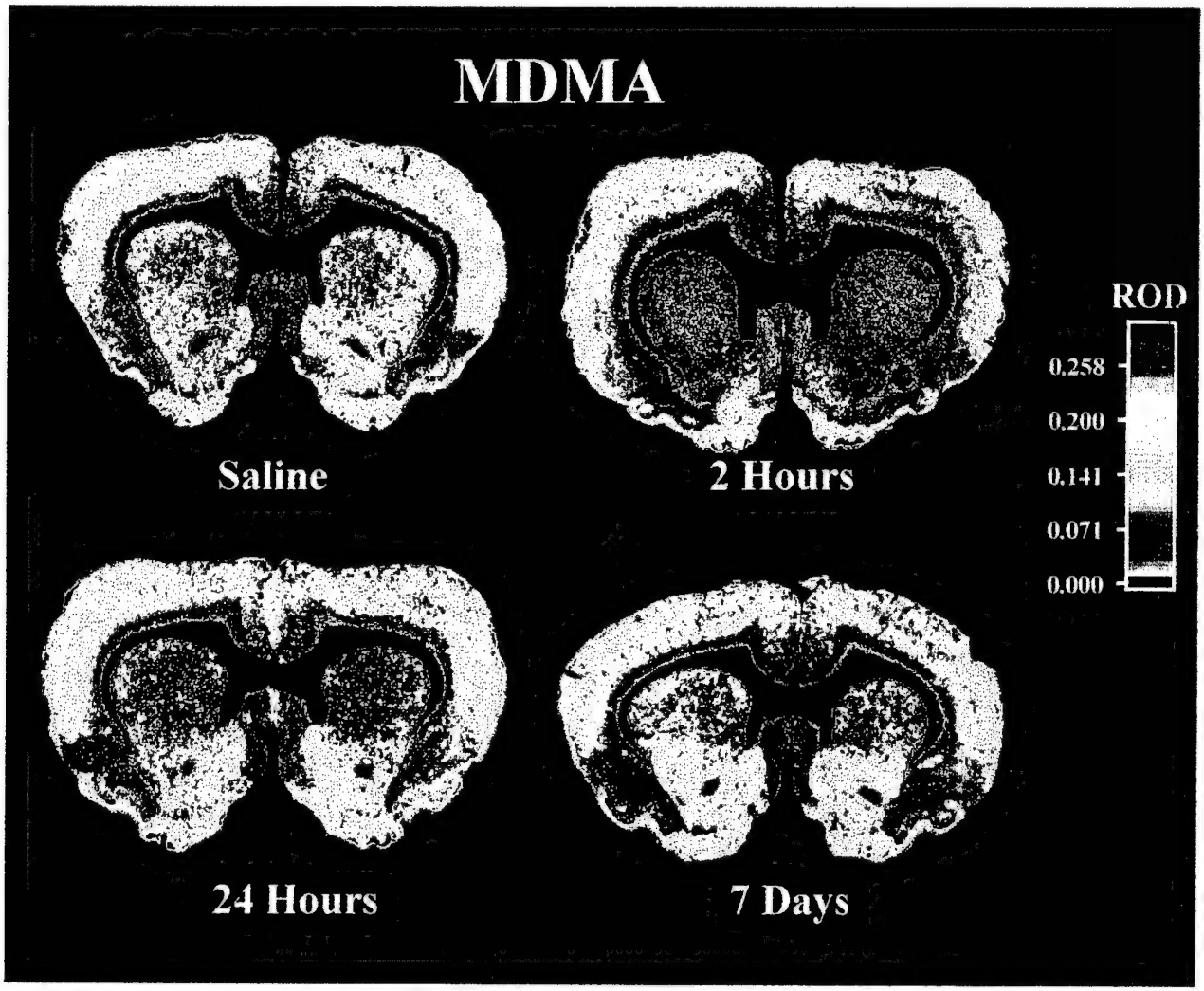

Fig. 2. Cytochrome oxidase histochemistry in representative coronal slices from animals treated with saline (top left) or MDMA (15 mg/kg) and killed 2 h (top right), 24 h (bottom left), or 7 days (bottom right) following the last dose of drug. Note the decrease in density of staining within the caudate and nucleus accumbens 2 h following MDMA administration (panel B) compared to a saline control rat (panel A).

pars reticulata were seen. Because boundaries between the nigral subregions (pars compacta and pars reticulata) were not always distinct in stained slices, cytochrome oxidase activity was determined for the entire substantia nigra.

Rectal temperatures rapidly increased following each injection of methamphetamine (10 mg/kg) (Fig. 4). In contrast, hyperthermia experienced by MDMA (15 mg/kg)-treated rats was stable across drug administration. Although administration of either drug produced hyperthermia when compared to saline-treated rats (peak temperature 37.6 ± 0.09°C), peak temperatures attained following methamphetamine administration (41.2 ± 0.17°C) were higher than those found following MDMA (39.6 ± 0.26°C) ($F(2,48) = 102$, $P < 0.01$).

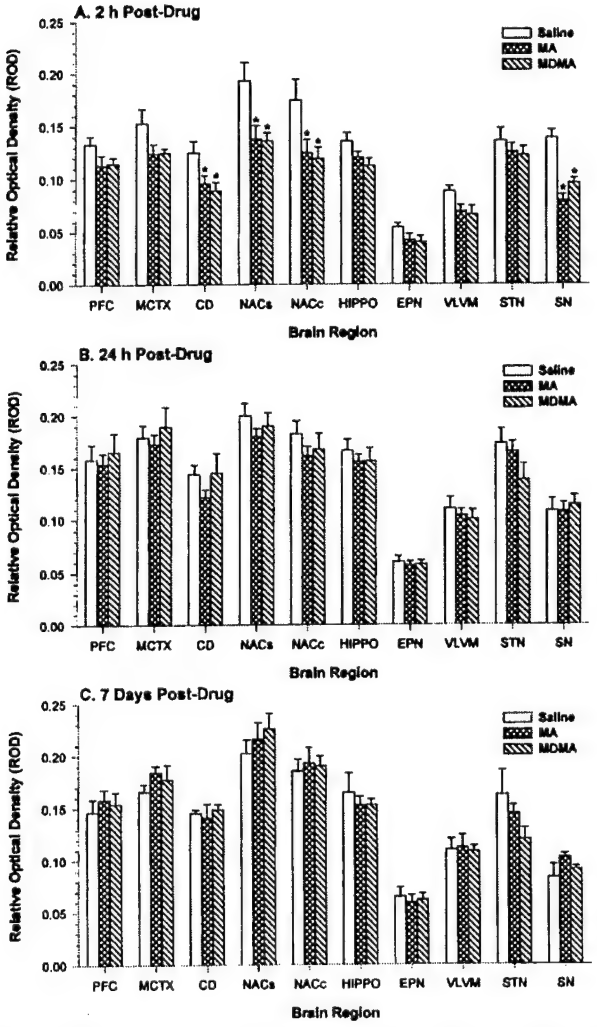

Fig. 3. Effect of repeated methamphetamine (10 mg/kg) or MDMA (15 mg/kg) on the density of cytochrome oxidase histochemical staining (ROD score) (A) 2 h ($n = 5$), (B) 24 h ($n = 7$–10), or (C) 7 days ($n = 5$–6) following the last dose of drug. Cytochrome oxidase activity varied across brain region in saline treated control animals. Cytochrome oxidase activity was significantly decreased 2 h following methamphetamine or MDMA administration in dopamine terminal regions (CD, NACs, NACc) and in the SN (* $P < 0.05$). No differences in cytochrome oxidase staining were found 24 h or 7 days post drug. Anatomical abbreviations are as follows: PFC, prefrontal cortex; MCTX, motor cortex; CD, caudate; NACs, nucleus accumbens shell; NACc, nucleus accumbens core; HIPPO, hippocampus; EPN, entopeduncular nucleus; VLVM, motor thalamus; STN, subthalamic nucleus; SN, substantia nigra.

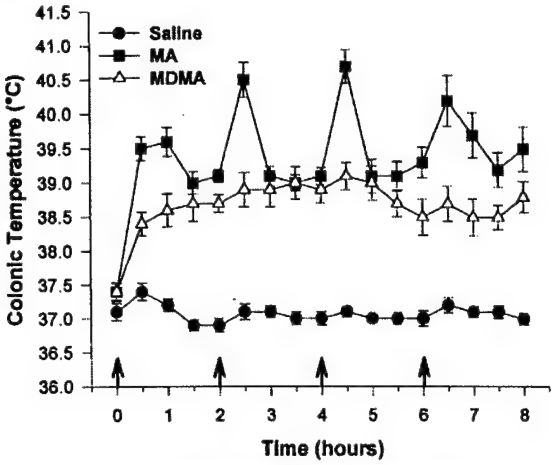

Fig. 4. Effect of methamphetamine (10 mg/kg) or MDMA (15 mg/kg) on core body temperature (°C). Systemic administration (arrows) produced a hyperthermic response compared to rats injected with saline vehicle ($n = 17$) ($P < 0.01$). Core body temperature peaked 30 min following each injection of methamphetamine ($n = 19$). In contrast, rats treated with MDMA ($n = 15$) had a hyperthermic response that remained fairly constant throughout the 8 h of drug administration.

4. Discussion

The acute administration of methamphetamine and MDMA resulted in a rapid and transient decrease in complex IV activity. Both methamphetamine and MDMA decreased cytochrome oxidase staining to a similar extent in the caudate, nucleus accumbens, and substantia nigra. These changes occurred within 2 h following the final drug injection and returned to basal levels within 24 h. No significant alterations in cytochrome oxidase activity were found in six other brain regions examined. Chan et al. (1994) reported that methamphetamine administration to mice (using the same dosing regimen), depleted ATP levels by 20% in the striatum, but not the hippocampus. In addition, ATP levels were depleted 1.5 h following methamphetamine administration, and returned to control values within 24 h (Chan et al., 1994). Thus, the inhibition of complex IV activity found in the current study correlates both temporally and anatomically with the ATP depletion that has been found previously to occur after high-dose methamphetamine administration. Together, these studies provide evidence that acute high-dose psychostimulant administration produces a rapid and transient disruption of metabolic processes that is regionally selective.

Several mechanisms could underlie the compromise in metabolic function that follows methamphetamine or MDMA administration. Evidence indicates that the release of nitric oxide and subsequent activation of the nitric oxide synthase pathway follow both methamphetamine and MDMA administration (Abekawa et al., 1996; Zheng and Laverty, 1998). Since nitric oxide is a known cytochrome oxidase inhibitor (Cleeter et al., 1994; Lizasoain et al., 1996), stimulant-induced activation of this pathway may directly inhibit complex IV activity. Additionally, psychostimulants may increase neuronal energy utilization through the sustained activation of monoamine transporters, hyperlocomotion, and the production of hyperthermia, all of which have been described as responses to psychostimulant administration (Huether et al., 1997). Since the majority of ATP in the neuropil is devoted to the maintenance of ion gradients and the restoration of the membrane potential following depolarization (Erecinska and Silver, 1989; Hevner et al., 1992; Wong-Riley, 1989), sustained activation following prolonged neurotransmitter release may lead indirectly to the depletion of substrates for the electron transport chain. Such a decrease in available precursors may slow or halt the production of ATP through a decline in complex IV activity.

Stimulant-induced increases in the extracellular concentrations of monoamines may also contribute to mitochondrial inhibition. Elevated extracellular dopamine may compromise mitochondrial function via autoxidation to form quinones and/or the enzymatic degradation of dopamine to form H_2O_2 and the generation of hydroxyl radicals (Graham et al., 1978; McLaughlin et al., 1998). This hypothesis is especially interesting given the finding that significant decreases in cytochrome oxidase activity were restricted to dopamine-rich brain regions (striatum, nucleus accumbens, and substantia nigra). Reactive oxygen species and dopamine-derived quinones are known to directly inhibit mitochondrial enzymes associated with energy production (Ben-Schachar et al., 1995; Yagi and Hatefi, 1987; Zhang et al., 1990). Although dopamine and 5-HT-mediated inhibition of energy production has not been demonstrated to occur in vivo, incubation of rat brain mitochondria in vitro with dopamine decreases State 3 (ATP-synthesis coupled) respiration, and incubation with dopamine–quinones increases State 4 respiration (Berman and Hastings, 1999). These studies indicate that reactive dopamine by-products may increase proton leakage across the mitochondrial membrane and inhibit the production of energy stores. Although Berman and Hastings (1999) reported that the L-DOPA-mediated decrease in State 3 respiration was not due to altered complex IV activity, L-DOPA does inhibit complex IV, but not complex I, activity in vitro (Pardo et al., 1995). In addition, Jiang et al. (1999) have recently reported that tryptamine-4,5-dione, a metabolite of 5-HT oxidation, inhibits cytochrome oxidase and NADH-coenzyme Q1 reductase in vitro by covalently modifying sulfhydryl groups on these enzyme complexes. These data indicate that the sustained release of 5-HT following psychostimulant administration also may contribute to the inhibition of mitochondrial function.

In the current study, the effects of high-dose psychostimulants on complex IV activity were rapid and transient. In contrast, studies using similar doses of methamphetamine and MDMA have found persistent depletions of dopamine and/or 5-HT (Shankaran et al., 1999; Stephans and Yamamoto, 1994; Yamamoto and Zhu, 1998). Although the time courses of metabolic inhibition and persistent nerve terminal loss are discordant, a rapid and transient disruption of mitochondrial function may produce neurotoxicity. For example, Chan et al. (1994) reported that inhibition of metabolism, by pretreatment with 2-deoxyglucose, exacerbated both the methamphetamine-induced ATP loss and long-term reduction of striatal dopamine content. Conversely, pretreatment with nicotinamide attenuated both the rapid decrease in striatal ATP and the lasting dopamine depletions following amphetamine administration (Wan et al., 1999). In addition, the intrastriatal perfusion of substrates for the electron transport chain (ubiquinone or nicotinamide) for several hours following methamphetamine administration attenuated the long-term loss of dopamine content, again linking a metabolic deficit with loss of monoamine nerve terminals (Stephans et al., 1998). Furthermore, the local inhibition of complex II via intrastriatal perfusion with malonate synergized with the local administration of methamphetamine to enhance dopamine toxicity compared to the perfusion of either drug alone (Burrows et al., 2000). These data indicate that a depletion of energy stores is critically linked with the neurotoxic effects of stimulant drugs. Although no direct measures of toxicity were made in the present study, the hyperthermic response to psychostimulant administration was comparable to that previously shown to be associated with lasting monoamine depletions (Bowyer et al., 1994; Che et al., 1995). Additional studies are needed to examine the possible relationship between the inhibition of complex IV activity and lasting monoamine depletions that can occur following methamphetamine or MDMA administration.

In conclusion, the rapid inhibition of metabolic function in dopamine-rich regions, as determined by a decrease in complex IV activity following methamphetamine and MDMA administration correlates with previous studies suggesting that psychostimulant administration compromises energy balance in the brain. This change in cytochrome oxidase activity could reflect protein turnover, a loss of enzyme function, or uncoupling of oxidative phosphorylation. Future studies are needed to determine the underlying mechanism of the transient loss of cytochrome oxidase activity. These studies add to the growing importance for understanding the consequences of psychostimulant administration in light of the recent evidence of dopamine terminal dysfunction in human methamphetamine abusers (McCann et al., 1998a,b; Wilson et al.,

1996) and the physiological abnormalities in humans who have used MDMA (Allen et al., 1993; Bolla et al., 1998; Morgan, 1999).

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Central Administration of Methamphetamine Synergizes with Metabolic Inhibition to Deplete Striatal Monoamines[1]

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ABSTRACT

These studies examined, in vivo, the effect of local intrastriatal perfusion of methamphetamine (MA) on dopamine (DA) and glutamate release in relation to changes in striatal DA and serotonin (5-HT) content measured 1 week after treatment. Interactions between the inhibition of energy metabolism and the direct perfusion of MA on long-term decreases in DA and 5-HT content also were investigated. MA (100 μM), the succinate dehydrogenase inhibitor malonate, or the combination of MA and malonate was reverse-dialyzed into the striatum for 8 h. The continuous local perfusion of MA alone increased DA release by 30-fold, similar to that seen after systemic administration, but did not increase glutamate or body temperature, and did not deplete neurotransmitter content. Malonate perfusion increased both DA and glutamate overflow, and dose dependently decreased DA content. 5-HT content was not as affected by malonate perfusions (200 mM malonate depleted DA by 66% and 5-HT by 40%). When MA was coperfused with 200 mM malonate, DA content was reduced by 80% and to a greater extent compared with malonate alone. Coperfusion of MA and 200 mM malonate did not enhance 5-HT loss. Overall, the present findings provide evidence that energy metabolism plays an important role in MA toxicity and that striatal dopaminergic terminals are more vulnerable than 5-HT terminals to damage after metabolic stress.

Methamphetamine (MA) is a psychostimulant that induces lasting depletions of striatal dopamine (DA) and serotonin (5-HT) content (Seiden et al., 1975, 1976; Ricaurte et al., 1980). Despite characterization of MA-induced toxicity over the past 25 years, it remains unknown how this drug of abuse damages the brain. There is evidence that MA increases both oxidative and metabolic stress, which may mediate the toxic effects of this drug. Systemic MA increases hydroxyl radical formation and leads to protein modification in the striatum (Giovanni et al., 1995; Yamamoto and Zhu, 1997; LaVoie and Hastings, 1999). In addition, energy use is increased after systemic MA, as evidenced by an immediate and sustained increase in the extracellular concentrations of lactate in the striatum (Stephans et al., 1998). Furthermore, ATP concentrations are depleted in brain regions susceptible to MA (Chan et al., 1994).

The excessive release of DA and glutamate have both been implicated in mediating the damage to DA terminals (Sonsalla et al., 1989; O'Dell et al., 1991; Stephans and Yamamoto, 1994). DA can contribute to oxidative damage via autoxidation to form quinones and/or increased enzymatic degradation to form the neurotoxic by-product H_2O_2 (Graham et al., 1978; McLaughlin et al., 1998). There also may be a relationship between the increase in glutamate release and MA-induced depletion of energy stores. Glutamate activation of NMDA receptors can increase nitric oxide synthase (Lizasoain et al., 1996). The subsequent production of nitric oxide can lead to the formation of reactive oxygen species (peroxynitrite) and to mitochondrial dysfunction by directly inhibiting complex IV of the electron transport chain, cytochrome *c* oxidase (Cleeter et al., 1994; Lizasoain et al., 1996). Conversely, local striatal perfusion with substrates for the electron transport chain at either complex I (nicotinamide), II, or III (ubiquinone) attenuate MA toxicity (Stephans et al, 1998).

The above-mentioned studies indicate that a disruption in mitochondrial function, in conjunction with oxidative stress, may mediate the toxic effects of MA. Metabolic inhibition induced by the reversible complex II inhibitor malonate preferentially damages DA versus γ-aminobutyric (GABA) acid terminals when infused into the striatum (Beal et al., 1993; Zeevalk et al., 1997). Similar to systemic MA, malonate infusions increase lactate production, diminish ATP stores, and deplete DA content (Beal et al., 1993; Albers et al., 1996). Infusion of malonate into the striatum is additive with systemic MA to produce an enhanced DA depletion (Albers et al., 1996), indicating a relationship between damage induced by metabolic inhibition following either striatal infusions of malonate or systemic MA.

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ABBREVIATIONS: MA, methamphetamine; DA, dopamine; 5-HT, serotonin, GABA, λ-aminobutyric acid; PE, polyethylene; AUC, area under the curve; HPLC, high performance liquid chromatography.

Although most studies have used systemic administrations of MA, this route of administration produces potent peripheral sympathetic effects such as hyperthermia, a known contributor to toxicity (Bowyer et al., 1994; Albers and Sonsalla, 1995). Consequently, interpretations regarding the specific mechanisms mediating the neurotoxic effects of MA are confounded by a variety of systemic effects produced by this drug. No studies to date have directly examined the effect of local intrastriatal perfusion of MA on DA and glutamate release in relation to long-term changes in striatal neurotransmitter content. Moreover, the interaction between inhibition of energy metabolism and direct perfusion of MA on decreases in DA and 5-HT content has not been investigated. The objectives of the present study were to: 1) examine the possible neurotoxic effects of the direct perfusion of MA into the striatum; and 2) determine whether manipulation of energy metabolism, by using the mitochondrial complex II inhibitor malonate, contributes to the long-term depletions of striatal DA and/or 5-HT content produced by MA.

Materials and Methods

Subjects

Male Sprague-Dawley rats (200–300 g; Zivic-Miller Laboratory, Allison Park, PA) were maintained on a 12-h light/dark cycle (lights on 6:00 AM) in a temperature- (22°C) and humidity-controlled environment. Subjects were initially housed two to three per cage and subsequently housed individually after surgery. Food and water were available ad libitum. All experiments were performed in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the local animal care and use committee.

Drugs and Reagents

The following drugs and chemicals were used in these studies: *d*-methamphetamine (Sigma Chemical Co., St. Louis, MO), malonic acid (Sigma Chemical Co.), Dulbecco's powdered medium (Sigma Chemical Co.), and methanol (optima) (Fisher Scientific, Pittsburgh, PA).

Experimental Design

Experiment 1. Local versus Systemic MA Administration. Rats (n = 21) were administered MA either systemically (i.p., four injections of 10 mg/kg over 8 h) or intrastriatally via reverse dialysis (continuous perfusion of 100 μM for 8 h) (see below for details). For local perfusion studies, one side of the striatum was perfused with MA and the other side was perfused with vehicle. Saline vehicle was injected systemically into additional rats. To determine the effects of local or systemic MA on core body temperature, colonic temperatures were monitored in some animals by use of an RET-2 copper-constantan thermocouple rectal probe (Physitemp Instruments, Inc., Clifton, NJ) and a TH-8 thermalert thermometer (Sensortek, Inc., Clifton, NJ). Colonic temperature was measured once an hour for 8 h, starting at either the first injection of MA (n = 5) or saline (n = 4), or at the initiation of striatal perfusion of MA (n = 4). Ambient temperature was maintained at 22 ± 0.5°C for the duration of the experiment. Extracellular concentrations of DA and glutamate were determined for all animals used in experiment 1. Tissue concentrations of DA were determined 7 days after local or systemic drug administration.

Experiment 2. Local Administration of MA and Interactions with Complex II Inhibition. Rats (n = 96) with dual striatal microdialysis probes (see below for details) were administered one of three concentrations of the complex II inhibitor malonate alone, via reverse dialysis, or in combination with MA (100 μM). In most cases, one striatum was perfused with Dulbecco's alone, and the other striatum was perfused with Dulbecco's solution containing MA (100 μM), malonate (50, 100, or 200 mM), the combination of MA and malonate, or Dulbecco's alone. Similar to the reverse dialysis of many other drugs, it was assumed that only a small percentage of drug would actually cross the semipermeable dialysis membrane. Therefore, the concentration of malonate actually reaching the tissue is much less than the concentration of drug in the perfusion medium. The doses of malonate were chosen to equate the degree of DA depletion produced by malonate with that found by others. In preliminary studies, we found that a malonate concentration of 200 mM in the perfusion medium produced a 50% depletion of DA content. Although the actual amount of malonate that was in the tissue after reverse dialysis was not determined, this degree of DA depletion is similar to that found by injecting 2 μl of 2 M malonate directly into the striatum (Albers et al., 1996).

Dialysates were collected and analyzed for extracellular concentrations of DA and glutamate in some animals perfused locally with vehicle (n = 12), MA (n = 13, from experiment 1), 200 mM malonate (n = 6), and 200 mM malonate plus MA (n = 7). For the remaining animals, drugs were administered by reverse dialysis but dialysate was not collected. All animals in experiment 2 were sacrificed 7 days after dialysis and striatal tissue content of DA and 5-HT was determined.

To determine the effects of the coperfusion of MA and malonate on core body temperature, colonic temperatures were monitored in a subset of animals as described above. Colonic temperature was measured once an hour for 6 h, starting at the initiation of striatal perfusion of MA plus 100 mM malonate (n = 4) or MA plus 200 mM malonate (n = 6). Ambient temperature was maintained at 22 ± 0.5°C for the duration of the experiment.

Surgery

Rats were anesthetized with a combination of xylazine (7 mg/kg i.m.) and ketamine hydrochloride (70 mg/kg i.m.). The skull was exposed and holes were drilled over the left and right striatum (AP, +1.2; ML, +3.2 mm from bregma) (Paxinos and Watson, 1986). A stainless steel guide cannula (21-gauge) was lowered through each hole and onto the dura and cemented in place with cyanoacrylate glue. The cannulas were secured to the skull with three stainless steel machine screws and cranioplastic cement. Animals were allowed at least 3 days of recovery before dialysis.

In Vivo Microdialysis

All dialysis probes were of a concentric flow design and were constructed as previously described (Yamamoto and Pehek, 1990). The length of the dialysis membrane (SpectraPor, 13,000 mol. wt. cutoff, 210 μm o.d.) was 4 mm. The dead volumes of all probes were calculated so as to synchronize the timing and initiation of drug perfusion with sample collection. On the day of dialysis, a 26-gauge stainless steel needle with a beveled tip that extended ~0.5 mm beyond the end of the guide cannula was used to puncture dura. The dialysis probes were inserted slowly through the guide cannulas and into the brain of awake rats to a premeasured distance so that the dialysis probes sampled the entire height of the lateral striatum. The probes were secured tightly to the cannulas with polyethylene (PE) 90 tubing that was previously glued to the probe. In addition, a layer of 5-min epoxy on the outside of the probe ensured that it remained in place. The dialysis probes were attached to a two-channel liquid swivel (Instech, Inc., Plymouth Meeting, PA) via PE 50 tubing (24 cm) allowing relatively unrestrained movement of the animal throughout the experiment. A spring tether connected the rat to the swivel and covered the PE 50 tubing. The probes were perfused with a modified Dulbecco's phosphate-buffered saline containing 138 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$, 1.5 mM KH_2PO_4, 1.2 mM $CaCl_2$, and 0.5 mM *d*-glucose, pH 7.4. For experiments where extracellular DA and glutamate were measured, dialysate was collected from the

outflow of the probe via microbore tubing (20 cm), the end of which was inserted into a 250-μl microcentrifuge tube that was clipped to the spring tether.

Dulbecco's alone, or in combination with other drugs (see experiments 1 and 2 described above), was perfused through the dialysis probes via a microinfusion pump (Harvard Apparatus, Holliston, MA) at a constant rate of 2 μl/min. For studies where extracellular DA and glutamate were determined, insertion of the probes was followed by a 3-h equilibration period before baseline sample collections. Dialysate samples were collected every 60 min for a 2- to 3-h baseline period, after which the perfusion medium was switched to one containing an experimental drug. Dialysate samples were collected every 60 min for an additional 8 h after the change in perfusion medium. For studies in which no dialysate was collected (i.e., probes used for the local perfusion of drugs only), the methods were identical with those described above with some exceptions. In those studies, insertion of the probes was immediately followed by 8 h of drug or vehicle perfusion. Although the dialysate was not saved, collection volume was monitored to ensure that probes were functioning properly.

Measurement of Extracellular DA and Glutamate

Extracellular concentrations of DA and glutamate were measured by high performance liquid chromatography (HPLC) with electrochemical detection as previously described (Donzanti and Yamamoto, 1988; Yamamoto and Davy, 1992). These neurotransmitters have been implicated in mediating striatal toxicity after a variety of insults, including high-dose MA treatment, transient ischemia, and heat stroke. DA was separated from metabolites with a reversed phase column (Phenomenex, Belmont, CA; C_{18}, 3-μm particle size, 2 × 100 mm) and a mobile phase consisting of 32 mM citrate, 54.3 mM sodium acetate, 0.074 mM disodium EDTA, 0.215 mM octyl sodium sulfate, and 3% methanol (v/v), pH 4.2. Flow rate was 0.2 ml/min. Detection was with an LC4C amperometric detector (Bioanalytical Systems, West Lafayette, IN) and a glassy carbon electrode (6-mm diameter) maintained at a potential of 0.6 V.

Dialysate also was assayed for glutamate by HPLC with electrochemical detection after precolumn derivatization with *o*-phthaldialdehyde. The derivatizing reagent was prepared by dissolving 27 mg of *o*-phthaldialdehyde in 1 ml of 100% methanol and 9 ml of 0.1 M sodium tetraborate (pH 9.4) to which 10 μl β-mercaptoethanol was added. This stock solution was then diluted 1:3 with the sodium tetraborate. A 10-μl aliquot of this reagent solution was then added to 20 μl of the dialysate. Derivatization was allowed to proceed for 2 min before injection onto the HPLC column. Glutamate was separated on a 3-μm C_{18} reversed phase column (Pheonomenex) and eluted with a 0.1 M sodium phosphate buffer (pH 6.4) containing 25% methanol and 50 mg/l EDTA. Detection was at a glassy carbon electrode maintained at +0.7 V by an LC 4B amperometric detector (Bioanalytical Systems). Flow rate was 0.4 ml/min.

Tissue DA and 5-HT Content

MA and/or malonate were perfused into the brain via reverse dialysis. Because it is not known how far away from the probe tract the locally administered drugs diffused, only the tissue adjacent to the probe site was analyzed for DA and 5-HT in hopes of maximizing the detection of local changes in neurotransmitter content. In addition, damage caused by the probe itself can affect neurotransmitter concentrations in the immediate vicinity of the tract (unpublished data). Therefore, in all studies we compared tissue adjacent to the probe that had been exposed to either drug or normal perfusion medium.

Seven days after dialysis, all rats were sacrificed by rapid decapitation, and brains were removed and quick-frozen in dry ice. Brains were sectioned on a cryostat at 40-μm intervals until the probe tract could be visualized. Then, a coronal slice ~400 μm in thickness was taken and the tissue ~0.5 mm to either side of the probe tract was dissected out under a microscope (40×). In initial studies, only DA was determined in tissue 1 week after drug administration. In later experiments designed to confirm our initial findings, both DA and 5-HT were determined in the same animals. Because no difference was found between DA concentrations in our initial experiments compared with our later experiments, the DA data were pooled across experiments.

Tissue samples were sonicated in 300 μl of cold 0.1 N $HClO_4$ and centrifuged at 12,000*g* for 10 min at 4°C. DA and 5-HT were separated on a C_{18} reversed phase column (Phenomenex; Prodigy 100 × 2 mm i.d., 3-μm particle size) with a mobile phase consisting of 200 mM sodium acetate, 12.5 mM citrate, 0.13 mM EDTA, and 5% methanol (v/v), pH 4.5. The column temperature was maintained at 34°C. The mobile phase was pumped at a flow rate of 0.4 ml/min. The analyates were quantitated with an electrochemical detector (EG&G Princeton Applied Research (Oakridge, TN) instrument model 400 electrochemical detector) by oxidation at a glassy carbon electrode (6-mm diameter) maintained at 0.6 V versus a Ag/AgCl reference electrode. Concentrations were expressed as picograms per microgram of protein. Protein was determined by the method of Bradford.

Statistics

DA and 5-HT content in striatal tissue were analyzed by two-factor ANOVA followed by Newman-Keuls where appropriate. DA content in rats treated with 200 mM malonate alone or in combination with MA were compared by Student's *t* test. Extracellular DA and glutamate concentrations, as determined by microdialysis, were analyzed by mixed two-factor ANOVA with repeated measures across time, followed by Newman-Keuls where appropriate. In experiment 1 (local versus systemic MA administration), the total DA response was expressed as area under the curve (AUC) and analyzed by Student's *t* test. In addition, the hyperthermic response to local or systemic MA was determined by AUC and analyzed by one-way ANOVA followed by Newman-Keuls where appropriate. For all analyses, significance was set as $\alpha = .05$.

Results

Experiment 1. Local Versus Systemic MA Administration

Extracellular DA and Glutamate Concentrations. Systemic administration of 10 mg/kg MA (i.p. every 2 h, total of four injections) increased extracellular concentrations of striatal DA (repeated-measures ANOVA, $F_{4,36} = 5.6, P < .01$) (Fig. 1). DA concentrations peaked 1 h after each injection and remained elevated throughout dialysis (8 h after the first injection). Local striatal perfusion of 100 μM MA produced a comparable increase in extracellular DA concentrations that peaked 1 h after the initiation of perfusion, and lasted throughout the remainder of the experiment (repeated-measures ANOVA, $F_{11,99} = 61.8, P < .01$). Although there were differences in the pattern of DA release after the continuous (local perfusion) or discontinuous (systemic i.p. injection) administration of MA, the overall DA response, measured as the AUC, was not significantly different between these two routes of drug administration (Student's *t* test, df = 16, $t = 1.3, P = .22$). Systemic administration of MA also resulted in a delayed rise in extracellular glutamate that began 6 h after the initial MA injection (repeated-measures ANOVA $F_{4,36} = 12.1, P < .01$) (Fig. 2). In contrast, local perfusion of the striatum with 100 μM MA for 8 h did not induce a similar rise in glutamate concentrations. Local or systemic administration of vehicle (Dulbecco's solution or normal saline, respectively) did not alter extracellular DA or glutamate concentrations (data not shown).

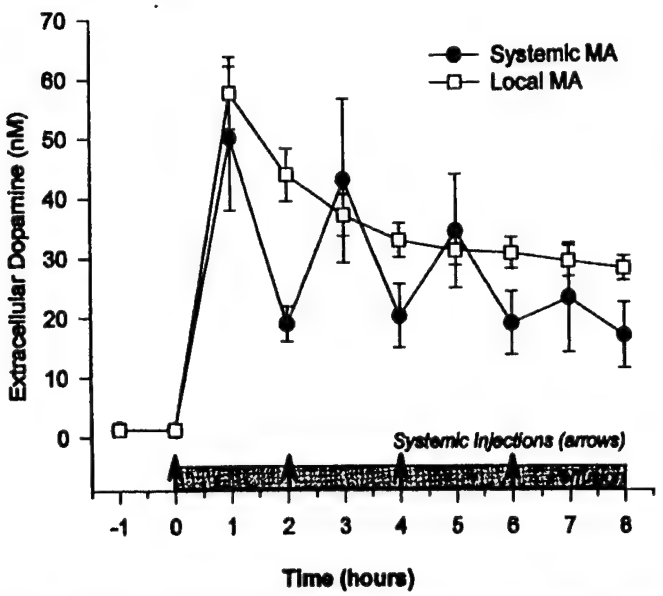

Fig. 1. Effect of local MA perfusion (100 μM) (n = 12) or systemic MA injections (10 mg/kg i.p.) (n = 6) on extracellular concentrations of DA in the striatum. Both routes of administration increased DA concentrations to a similar degree, but the pattern of release depended on the route of administration. DA concentrations increased 1 h after each systemic injection of MA (arrows), whereas local perfusion of the drug produced a peak effect within1 h that gradually declined over the 8-h perfusion period (shaded bar). The overall DA response (AUC) did not differ between the two groups.

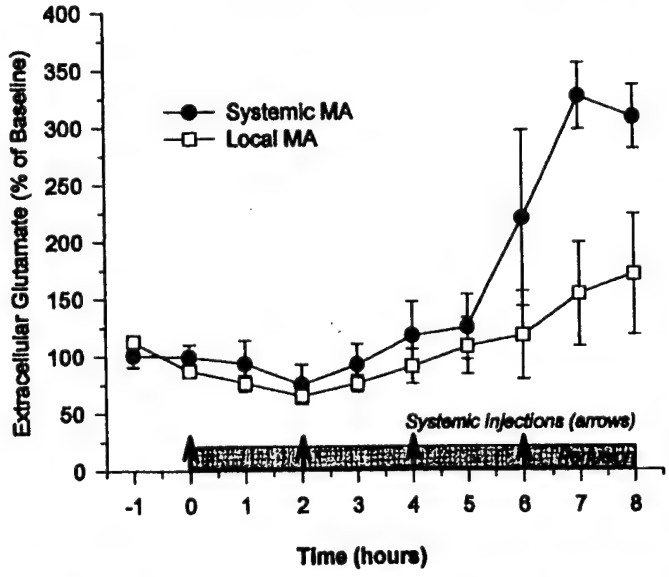

Fig. 2. Effect of local MA perfusion (100 μM) (n = 12) or systemic MA injections (10 mg/kg i.p.) (n = 5) on extracellular concentrations of glutamate in the striatum. Systemic injection of MA (arrows) produced a delayed rise in extracellular glutamate concentrations beginning 6 h after the initial dose ($P < .01$). Local perfusion of MA for 8 h (shaded bar) did not increase glutamate concentrations compared with baseline. Data were expressed as a percentage of baseline due to differences in basal concentrations of glutamate between experiments.

Tissue DA Content. One week after systemic administration of 10 mg/kg MA (i.p. every 2 h, total of four injections), striatal DA content was decreased by 70% (Fig. 3). In contrast, local MA perfusion for 8 h did not alter striatal DA content (mixed two-factor ANOVA interaction $F_{1,38} = 14.7$, $P < .01$).

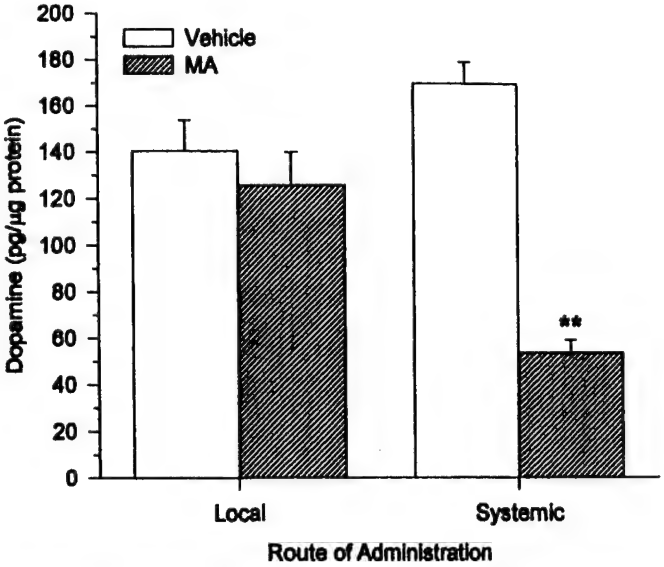

Fig. 3. Effect of local MA perfusion (100 μM) (n = 13) or systemic MA injections (four injections of 10 mg/kg i.p.) (n = 8) on DA concentrations the striatum. Rats were sacrificed 7 days after drug administration. Local perfusion of MA for 8 h did not alter DA tissue content, whereas systemic administration decreased DA concentrations by 70% (**$P < .01$ by Newman Keuls).

Hyperthermic Response. Systemic administration of MA (10 mg/kg i.p., four injections) produced a hyperthermic response (peak temperature 40.1 ± 0.5°C) (Fig. 4). Local striatal perfusions with MA for 8 h did not elevate core body temperature (peak temperature 37.8 ± 0.1°C) compared with that of rats that were given systemic saline (peak temperature 37.8 ± 0.2°C). Data (AUC) were analyzed by one-way ANOVA ($F_{2,10} = 44.1$, $P < .01$) followed by Newman-Keuls where appropriate.

Experiment 2. Local Administration of MA and Interactions with Complex II Inhibition

Extracellular DA and Glutamate Concentrations. The perfusion of malonate had a significant effect on MA-induced DA release over the course of the perfusion period (two-factor ANOVA, drug × time interaction $F_{27,150} = 4.5$, $P < .01$). Perfusion of 200 mM malonate via reverse dialysis resulted in a rapid increase in extracellular DA that peaked 1 h after initiation of drug infusion and rapidly returned to basal concentrations despite the continuing presence of drug in the perfusion medium (Fig. 5). In contrast, local perfusion of 100 μM MA alone resulted in a sustained rise in extracellular DA that lasted throughout the duration of the perfusion (data are regraphed from Fig. 1 to compare these concentrations with changes induced by the local perfusion of malonate). The combined administration of MA and 200 mM malonate induced a rapid and dramatic increase in extracellular DA concentrations (>60-fold) that peaked 1 h after initiation of drug infusion and returned to basal concentrations despite the continuing presence of both drugs in the perfusion medium.

Malonate, but not MA perfusion increased the extracellular concentration of glutamate over time (two-factor ANOVA, drug × time interaction $F_{27,120} = 16.2$, $P < .01$). Perfusion of 200 mM malonate via reverse dialysis resulted in a rapid and sustained increase in extracellular glutamate that was maximal 2 h after initiation of drug infusion (Fig. 6). In contrast,

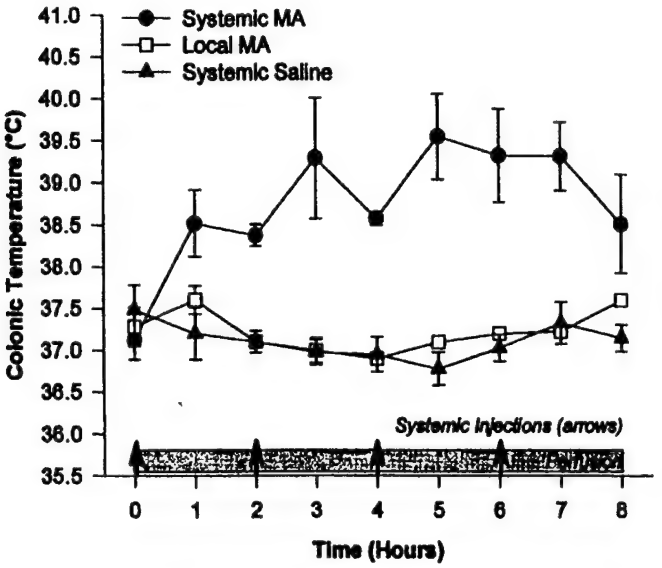

Fig. 4. Effect of local MA perfusion (100 μM) or systemic MA injections (four injections of 10 mg/kg i.p.) on core body temperature (°C). Local perfusion of MA for 8 h (n = 4) (shaded bar) did not alter colonic temperature compared with saline-treated rats (n = 4), whereas systemic administration (n = 5) (arrows) produced a hyperthermic response (P < .01).

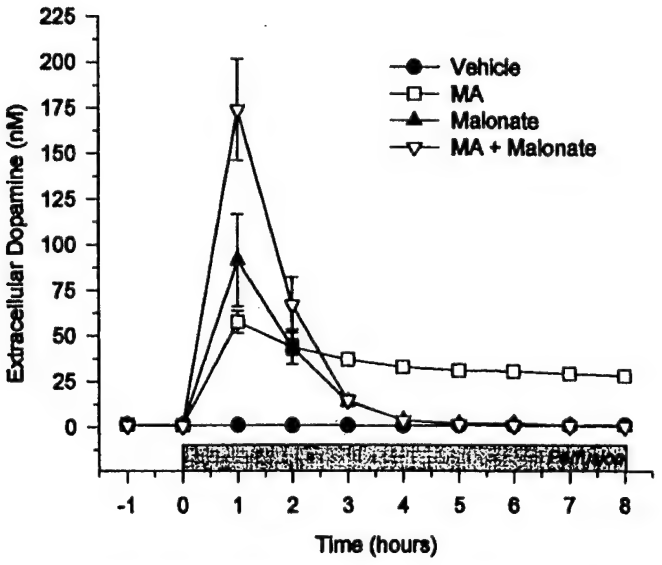

Fig. 5. Effect of local perfusion (shaded bar) of vehicle, MA (100 μM), malonate (200 mM), or the combination of MA and malonate on extracellular concentrations of DA in the striatum. Perfusion of MA alone (n = 12) produced a sustained increase in extracellular DA concentrations (regraphed from Fig. 1). Perfusions of 200 mM malonate alone (n = 6) or in combination with MA (n = 7) produced a rapid and transient increase in DA concentrations. The peak response occurred 1 h after initiation of perfusion and was greatest after the combination of MA and malonate infusion (P < .01). DA concentrations were not altered in vehicle-perfused striata (n = 12).

local perfusion of 100 μM MA did not alter extracellular glutamate concentrations (data are regraphed from Fig. 1). The combined administration of MA and 200 mM malonate induced a sustained increase in extracellular glutamate concentrations that was nearly identical with the response induced by malonate alone.

To determine whether combined drug infusions induced a state of hyperthermia, core body temperature was monitored

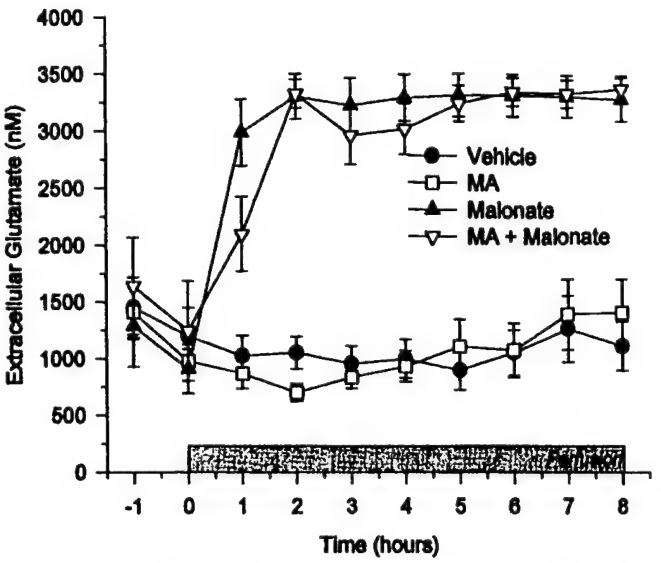

Fig. 6. Effect of local perfusion (shaded bar) of vehicle, MA (100 μM), malonate (200 mM), or the combination of MA and malonate on extracellular concentrations of glutamate in the striatum. Perfusion of MA alone (n = 12) did not increase glutamate concentrations (regraphed from Fig. 2). Local infusion of malonate, alone (n = 5) or in combination with MA (n = 8), produced a rapid and sustained increase in extracellular concentrations of glutamate (P < .01) that lasted the duration of the 8-h experiment. Glutamate concentrations were not altered in vehicle-perfused striata (n = 13).

in a subset of animals that received local infusions of MA plus 100 mM malonate (n = 4) or 200 mM malonate (n = 6). Colonic temperature did not change from basal concentrations during the 6 h after initiation of drug perfusion (MA plus 100 mM malonate: basal temperature = 37.0 ± 0.23°C, peak temperature = 37.7 ± 0.48°C; MA plus 200 mM malonate: basal temperature = 36.9 ± 0.10°C, peak temperature = 37.4 ± 0.18°C).

Tissue DA and 5-HT Content. Perfusion with 50 mM malonate alone or in combination with MA did not deplete DA concentrations (109 ± 11.36 versus 112 ± 8.38 ng DA/pg protein, respectively). In contrast, perfusion of higher concentrations of malonate alone (100 or 200 mM) produced a lasting depletion (>50%) of striatal DA content (Fig. 7). Perfusion of MA alone did not alter striatal DA content (data regraphed from Fig. 3). However, the combined perfusion of MA with increasing concentrations of malonate depleted DA content to a greater extent compared with malonate alone in the absence of MA (interaction $F_{2,36}$= 8.8, P < .01). In particular, the combined perfusion of 200 mM malonate plus MA produced a greater depletion of DA content compared with 200 mM malonate alone (80 versus 66% depletion, respectively).

Intrastriatal perfusion of 100 μM MA did not deplete striatal 5-HT content (Fig. 8). Similarly, perfusion of 100 mM malonate alone or in combination with MA did not decrease striatal 5-HT content. However, 200 mM malonate did decrease 5-HT content by 42% when examined 7 days after drug administration. Tissue concentrations of 5-HT 7 days after the combined perfusion of 200 mM malonate and MA were similar to those recorded after malonate alone (interaction $F_{2,18}$= 7.9, P < .01).

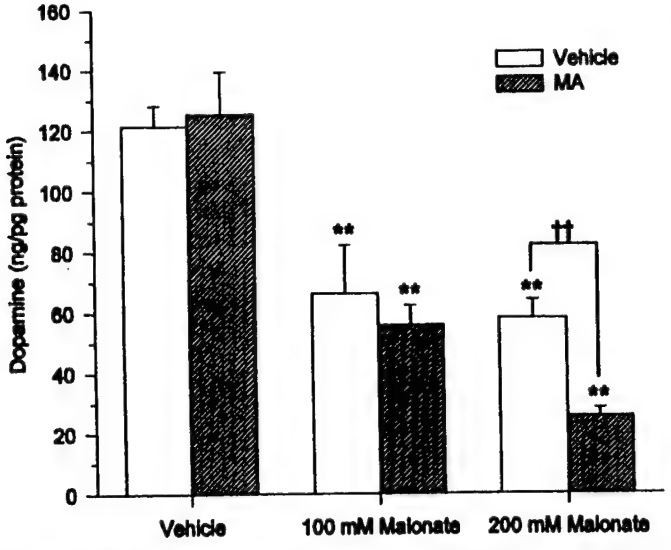

Fig. 7. Effect of local perfusion of vehicle, MA (100 μM), malonate (100 or 200 mM), or the combination of MA and malonate on tissue concentrations of DA in the striatum. Rats were sacrificed 7 days after drug administration. MA alone (n = 13) had no effect on DA content (regraphed from Fig. 3) compared with striata perfused with vehicle (n = 70). Malonate alone (100 mM, n = 7 or 200 mM, n = 26) decreased striatal DA content (**P < .01 versus respective vehicle control by Newman Keuls). Overall, malonate depleted DA to a greater extent when combined with MA compared with malonate alone without MA (**P < .01 versus respective vehicle control, by Newman Keuls). MA plus 100 mM malonate (n = 9) and 100 mM malonate alone depleted DA content in a similar manner. MA plus 200 mM malonate (n = 25) depleted DA content to a greater extent than 200 mM malonate alone (††P < .01 by Student's t test, df = 49, t = 4.3).

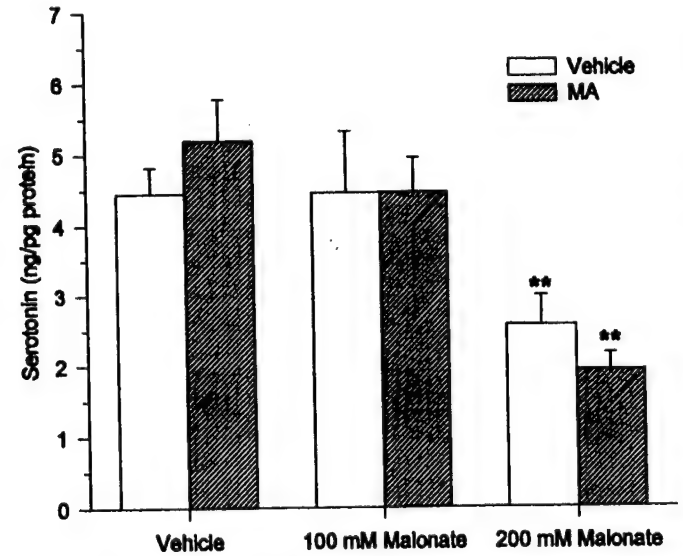

Fig. 8. Effect of local perfusion of vehicle, MA (100 μM), malonate (100 or 200 mM), or the combination of MA and malonate on tissue concentrations of 5-HT in the striatum. Rats were sacrificed 7 days after drug administration. Local perfusion of MA (n = 4), 100 mM malonate (n = 7), or the combination of MA and 100 mM malonate (n = 9) had no effect of striatal 5-HT content compared with striata perfused with vehicle (n = 45). Perfusion with 200 mM malonate alone (n = 15) or in combination with MA (n = 11) depleted 5-HT concentrations in a similar manner (**P < .01 versus respective vehicle control by Newman Keuls).

Discussion

The acute and long-term effects after the systemic administration of MA were compared with the local intrastriatal perfusion of MA. Both routes of administration acutely increased DA release, but only the systemic administration of MA increased extracellular concentrations of glutamate. Furthermore, only the systemic route of administration produced lasting decreases in striatal DA content. In contrast, intrastriatal perfusion of the reversible succinate dehydrogenase inhibitor malonate acutely increased extracellular concentrations of DA and glutamate, and dose dependently depleted striatal monoamine content. Moreover, the combined perfusion of MA with malonate (200 mM) enhanced the long-term depletion of striatal DA compared with malonate alone, but did not further deplete 5-HT.

The present study extends previous findings illustrating that other amphetamine derivatives are not neurotoxic when administered centrally (Berger et al., 1990; Paris and Cunningham, 1992). The current results offer a mechanistic explanation for the differences observed after the central versus systemic administration of MA by comparing the acute effects on DA and glutamate release and the long-term changes in tissue content. The lack of a long-term effect on DA tissue content after the local perfusion of MA is inconsistent with the hypothesis that elevated extracellular DA is the primary contributor to toxicity via autoxidation and/or increased enzymatic degradation of DA to form quinones and the H_2O_2-dependent generation of hydroxyl radicals (Graham et al., 1978; McLaughlin et al., 1998). These results are, however, consistent with the findings of LaVoie and Hastings (1999) that increased extracellular DA concentrations are not always correlated with MA-induced damage to DA terminals. The inability of locally applied MA to increase extracellular glutamate may explain the lack of a long-term effect on DA content, and further implicate the importance of glutamate in mediating terminal damage caused by systemic MA (Sonsalla et al., 1991; Abekawa et al., 1994; Stephans and Yamamoto, 1994).

Selective lesioning of striatal output neurons also blocks MA toxicity (O'Dell et al., 1994), indicating that activation of the extrapyramidal motor loop may be a critical step in mediating the excitotoxic effects of MA. This is consistent with the observation that MA-induced glutamate release is dependent on DA receptor activation because D2 antagonism with haloperidol attenuates the increase in extracellular glutamate concentrations after systemic MA (Stephans and Yamamoto, 1994). However, even in the presence of high extracellular concentrations of DA during locally applied MA, extracellular glutamate concentrations in striatum were unaltered. Given the large volume of the striatum, the relatively discrete area perfused with MA may not have been sufficient to influence the number of output neurons necessary to activate the extrapyramidal loop and enhance corticostriatal glutamate overflow. In addition, although the degree of DA release was similar after either systemic or local drug perfusion, the relative concentration of MA in the striatum after these different routes of administration is not known. The concentration of MA in the brain that is needed to enhance DA release may not be the same as that required to increase glutamate overflow.

Hyperthermia also has been implicated in long-term DA depletions produced by the systemic administration of MA (Bowyer et al., 1994; Albers and Sonsalla, 1995). Because the local striatal perfusion of MA does not alter core body temperature, the lack of effect of locally applied MA on DA

content examined 7 days later could be attributed to the absence of hyperthermia. Although hyperthermia contributes to the toxic effects of MA, hyperthermia alone is not sufficient to deplete DA concentrations (Burrows and Meshul, 1999). One mechanism by which hyperthermia could contribute to the pharmacological properties of MA is through a decrease in mitochondrial function and a subsequent depletion of energy stores. In addition, hyperthermia (40°C) increases the toxicity of locally perfused glutamate (Suehiro et al., 1999), indicating that hyperthermia may exacerbate the excitotoxic effects of MA. Thus, the lack of an increase in body temperature and the absence of a rise in extracellular glutamate may explain the absence of neurotoxicity after the local administration of MA.

Hyperthermia and glutamate release contribute to metabolic stress and may be key mediators in the toxic effects of MA. The present study examined the effects of the specific inhibition of mitochondrial function on DA and glutamate release as well as the contribution of this manipulation to the acute and long-term effects of local MA perfusion. Striatal perfusions of the reversible succinate dehydrogenase inhibitor malonate rapidly increased extracellular DA and glutamate but did not alter body temperature. The increase in extracellular glutamate is consistent with a previous report of enhanced glutamate release after the intrastriatal infusion of malonate (Messam et al., 1995). Although the mechanism mediating malonate-induced glutamate and DA release is unknown, the depletion of ATP stores by malonate (Beal et al., 1994) and the subsequent breakdown of the Na^+/K^+ gradient via the inhibition of Na^+/K^+ ATPase, may lead to carrier-mediated release of these neurotransmitters (Westerink et al., 1989; Zeevalk and Nicklas, 1991). An ATP-dependent mechanism also may explain the transient increase in DA release in the presence of malonate. DA release could occur initially via a NA^+-dependent reversal of the DA transporter that is followed by a cessation of transporter-mediated release as ATP concentrations are depleted due to the inhibition of mitochondrial respiration by malonate.

Intrastriatal infusions of malonate produced a dose-dependent depletion of striatal DA and 5-HT content. Although 100 mM malonate was sufficient to deplete DA concentrations, a 2-fold higher concentration of malonate was needed to deplete 5-HT. Moreover, the magnitude of 5-HT depletion was not as large as the depletion of DA content. Although intrastriatal injection of malonate preferentially damages DA compared with GABA terminals (Zeevalk et al., 1997), the differential effects of malonate on striatal 5-HT content, in vivo, have not been reported previously. In cultured mesencephalic neurons and synaptosomal preparations, other inhibitors of oxidative phosphorylation decrease DA uptake to a greater degree compared with uptake of GABA, 5-HT, and norepinephrine (Marey-Semper et al., 1993). These data are consistent with the conclusion that DAergic neurons are inherently more sensitive to damage mediated by metabolic stress. In addition, vulnerability to mitochondrial inhibition may underlie DA-specific neurodegenerative disorders such as Parkinson's disease (DiMauro, 1993). Although the etiology of enhanced vulnerability to toxicity is not known, the ability of DA to autoxidize, combined with the enzymatic oxidation of DA to form H_2O_2, may lead to elevated concentrations of intracellular reactive oxygen species that render DA neurons more vulnerable to metabolic inhibition or excitotoxic events.

The combined perfusion of malonate with MA produced a greater depletion of striatal DA content compared with malonate alone. This synergistic effect was selective for DA because the combined perfusion of MA and malonate did not enhance 5-HT depletion compared with the depletion produced by malonate alone. In fact, 5-HT terminals appear to be less vulnerable than DA terminals to toxicity resulting from metabolic inhibition. DA depletions after a lower concentration of malonate (100 mM) with or without MA is similar to the magnitude of 5-HT depletions observed with a higher dose of malonate (200 mM) (Figs. 7 and 8). Higher concentrations of MA and/or malonate may further enhance 5-HT depletion.

There are several mechanisms by which MA perfusion might enhance damage to DA terminals after metabolic inhibition. The increased extracellular glutamate concentrations after perfusion of malonate may be a critical factor mediating MA-induced DA depletion. Because glutamate is not increased after intrastriatal MA alone, but malonate itself induces the release of glutamate, high extracellular concentrations of glutamate may be necessary for MA to produce lasting depletions of DA content. An increase in the intracellular concentrations of reactive oxygen species, resulting from the enhanced release of glutamate and DA after the combined perfusion of MA and malonate, also may mediate the synergistic effects of the depletion of DA content. Although LaVoie and Hastings (1999) have suggested that such intracellular changes in DA and DA-derived reactive oxygen species are more important than extracellular DA in mediating damage to DA terminals, increased extracellular DA is necessary, but perhaps not sufficient, for toxicity to occur (Wagner et al., 1983; Sonsalla et al., 1986; Stephans and Yamamoto, 1994). Thus, enhanced DA efflux, in the presence of high extracellular glutamate, may be responsible for the synergistic actions of MA and malonate to deplete striatal DA content.

In summary, the present study used the local perfusion of MA with or without the local perfusion of a mitochondrial inhibitor to elucidate the acute pharmacological effects that mediate the long-term damage to monoamine neurons produced by MA. These findings are consistent with the conclusion that enhanced glutamate overflow and inhibition of energy metabolism, possibly due to hyperthermia, are important components that converge to mediate the neurotoxicity of MA. Although the local perfusion of MA alone increased DA release, the combination of MA with malonate produced an increase in glutamate release and an additive increase in DA overflow. These combined effects could result in the synergistic enhancement of the long-term depletion of DA content. The neuroprotection afforded by the perfusion of substrates of mitochondrial enzymes after systemic MA administration (Stephans et al., 1998) is additional evidence that a depletion of energy stores mediates MA-induced damage to DA neurons. Further studies are needed it determine whether hyperthermia produced by the systemic administration of MA contributes to the lasting depletion of tissue DA (Bowyer et al., 1994) via the inhibition of mitochondrial respiration and the depletion of ATP stores (Madl and Allen, 1995). Regardless, the present findings are evidence that energy metabolism plays an important role in MA toxicity

and that striatal DAergic terminals are more vulnerable than 5-HT terminals to damage after metabolic stress.

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Acute and Long-Term Effects of 3,4-Methylenedioxymethamphetamine on Serotonin and Dopamine: Interactions with Inhibition of Energy Metabolism and Comparisons to Methamphetamine

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Running Title: MDMA Perfusion and Mitochondrial Inhibition

List of Abreviations

MDMA – 3,4-methylenedioxymethamphetamine

METH – methamphetamine

MAL – Malonate

5HT – Serotonin

AUC – Area under the curve

ANOVA – Analysis of variance

PKC – protein kinase C

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Abstract

The acute and long-term effects of the local perfusion of 3,4-methylenedioxymethamphetamine and the interaction with the mitochondrial inhibitor malonate were examined in the striatum and compared to a similar perfusion of methamphetamine. 3,4-methylenedioxymethampetamine, methamphetamine, malonate, or the combination of malonate with 3,4-methylenedioxymethamphetamine or methamphetamine was reverse dialyzed into the striatum for 8 hours via a microdialysis probe while extracellular dopamine and serotonin were measured. One week later, tissue immediately surrounding the probe was assayed for dopamine and serotonin tissue content. The local perfusion of 3,4-methylenedioxymethamphetamine and methamphetamine increased dopamine and serotonin release but did not produce long-term tissue depletions of dopamine or serotonin. Malonate also increased both dopamine and serotonin release but, in contrast to the amphetamines, only produced long-term depletions in dopamine. The combined perfusion of 3,4-methylenedioxymethampetamine/malonate or methamphetamine/malonate synergistically increased the release of dopamine and serotonin and produced a long-term depletion of dopamine in tissue. Depletions of serotonin concentrations in tissue were only observed following perfusion with 3,4-methylenedioxymethamphetamine/malonate. These results support the conclusion that dopamine, compared to serotonin, neurons are more susceptible to mitochondrial inhibition. Moreover, malonate interacts with the toxic effects of 3,4-methylenedioxymethamphetamine and methamphetamine to damage dopamine neurons. The effects of 3,4-methylenedioxymethamphetamine in combination with malonate on serotonin neurons suggest a role for bio-energetic stress in 3,4-methylenedioxymethamphetamine-induced toxicity to serotonin neurons. Overall, these results highlight the importance of energy balance

to the function of dopamine and serotonin neurons and to the toxic effects of MDMA and methamphetamine.

Key Words: Malonate, mitochondrial inhibition, microdialysis

3,4-Methylenedioxymethamphetamine (MDMA) is a substituted amphetamine that has recently gained popularity among drug users.[17] Although MDMA is structurally similar to another drug of abuse, methamphetamine (METH), MDMA produces different pharmacological effects.[38] Specifically, MDMA selectively targets serotonin (5HT) neurons; it produces a selective long-term depletion of rat brain 5HT [14,34,41] and decreases tryptophan hydroxylase[41] and 5-HT uptake sites[3] without causing changes in dopaminergic neuronal markers. METH also produces depletions in serotonin tissue concentrations[32] as well as long-term decreases in markers of dopaminergic function including loss of dopamine uptake sites,[42] decreases in tyrosine hydroxylase activity[12] and dopamine content.[32,33] The exact mechanisms that mediate the differential toxic effects of these drugs on dopamine and/or 5HT neurons remain to be elucidated.

There is evidence to suggest that alterations in energy metabolism might be involved in the toxic actions of the amphetamines. The systemic administration of MDMA and METH produce hyperthermia, a critical mediator of the neurotoxicity produced by these drugs.[6,21] Moreover, both hyperthermia and d-amphetamine produce a metabolic pattern indicative of a disturbed balance between energy production and utilization.[26,27] Bio-energetic stress may contribute to MDMA toxicity as evidenced by an MDMA-induced increase in glycogen breakdown in astroglial rich cell cultures.[31] Furthermore, METH produces a loss of striatal ATP[8] and substrates of mitochondrial energy metabolism attenuate the neurotoxic effects of METH.[40] Few studies to date have examined whether bio-energetic stress differentially affects 5HT and dopamine neurons and interacts with the pharmacological action of MDMA and METH to produce long-term depletions of 5HT and/or dopamine. We have recently shown that METH synergizes with mitochondrial inhibition to deplete striatal dopamine but not serotonin tissue

concentrations.[7] There are, however, no studies that examine the interaction of mitochondrial inhibition with MDMA. Subtle differences in the pharmacological selectivity between MDMA and METH may interact with the inherent vulnerabilities of 5HT and dopamine neurons to bio-energetic stress and contribute to the different neurotoxic profiles of these drugs.

The current study examined the role of bio-energetic stress in mediating the differential effects of a local perfusion of MDMA in comparison with METH on striatal 5HT and dopamine neurons. Malonate (MAL) was used to produce bio-energetic stress because it inhibits succinate dehydrogenase and causes a decrease in striatal ATP.[1] Although MAL has been shown to damage dopamine neurons[45] and synergizes with METH to deplete striatal dopamine,[7] nothing is known about its interactions with MDMA and its effects on 5HT terminals. Therefore, MAL was used in conjunction with MDMA and METH to examine the interaction between the inhibition of electron transport and the acute and long-term effects of the amphetamines on striatal 5HT and dopamine terminals.

EXPERIMENTAL PROCEDURES

Animals

Male Sprague-Dawley rats (200-290g, Zivic-Miller, Allison Park, PA) were housed 3 per cage and provided with food and water *ad libitum* in a temperature controlled environment (20-22° C) with a 12h:12h light-dark cycle. After the surgical procedure, rats were housed individually for the duration of the experiments. All experiments were performed in strict accordance with the *NIH Guide for the Care and Use of Laboratory Animals* and were approved by the local animal care and use committee.

Drugs

The following reagents were used: *d*-methamphetamine (Sigma Chemical Co., St Louis, MO), 3,4-methylenedioxymethamphetamine (NIDA), malonic acid (Sigma Chemical Co., St. Louis, MO), Dulbecco's powdered medium (Sigma Chemical Co., St. Louis, MO)

Surgical Procedures

Rats were anaesthetized with xylazine/ketamine (6 mg/kg, 70 mg/kg) and placed in a stereotaxic apparatus. The skull was exposed and a stainless steel guide cannula (11 mm) with a stylet obturator was lowered onto the dura directly above the striatum on each side of the brain (AP:+1.2; ML:$\pm$3.2).[30] The two cannulae were secured in place by cranioplastic cement, 3 stainless steel skull screws, and cyanoacrylate glue. Rats were allowed to recover for at least 3 days prior to microdialysis.

In Vivo Microdialysis

On the day of dialysis, the obturators were removed from the guide cannulae and microdialysis probes slowly inserted through the cannula into the brain of the awake rat. The probes were a concentric flow design and were constructed as previously described.[44] The probes were designed so that the dialysis membrane of 4.0 mm (SpectraPor, 13,0000 MV cutoff; 210 μm O.D.) sampled from the entire dorso-ventral extent of the lateral striatum. The probes were connected via spring-covered PE-50 tubing to a dual channel swivel (Instech, Plymouth Meeting, PA) that allowed for relatively unrestrained movement of the animal. For experiments in which extracellular dopamine and 5HT were measured, dialysate was collected into 250 μl tubes clipped to the tether. The probes were perfused with modified Dulbecco's phosphate-buffered saline (138 mM, 2.1 mM KCl, 0.5 mM $MgCl_2$, 1.5 mM KH_2PO_4, 8.1 mM $NaHPO_4$, 1.2 mM $CaCl_2$, and 0.5 mM d-glucose, pH 7.4), which was pumped at a flow rate of 2.0 μl/min (Syringe infusion pump, Harvard Apparatus, Holliston, MA). In experiments where extracellular dopamine and 5HT were determined, Dulbecco's alone was perfused for a 3-hour equilibration period, and then 2 sixty minute baseline samples were collected. The perfusion medium was then switched on one side of each animal to Dulbecco's containing either MDMA (100 μM), METH (100 μM), MAL (100 mM) or the combination of either MDMA/MAL or METH/MAL. The perfusion continued for an additional 8 hours. Samples were collected every 60 min. In other experiments, no dialysate was collected, (i.e., probes were used only for the local perfusion of drugs). The methods in these experiments were identical to those described above except that perfusion of drug occurred immediately following insertion of the probe and continued for 8-hours. The dialysate was not collected but flow was monitored to insure that the probes were functional.

Body temperatures (colonic temperature) were monitored throughout some of the dialysis experiments. Baseline body temperatures were measured 30 minutes prior to the perfusion of drug, 30 minutes after drug perfusion began, and then every 60 minutes for the next 4 hours. A final measurement was recorded 60 minutes prior to the termination of the perfusion.

Determination of Extracellular Serotonin and Dopamine

Each dialysate sample was divided and assayed for 5HT or dopamine by high performance liquid chromatography (HPLC) with electrochemical detection. Samples (20 µl) were injected onto a 3 µm C18 reverse-phase column (100 x 2.0 mm, Phenomenex, Torrance CA). Dopamine and 5HT were eluted with a mobile phase consisting of 32 mM citric acid, 54.3 mM sodium acetate, 0.074 mM ethylenediaminetetraacetic acid (Na EDTA), 0.215 mM octyl sodium sulfate and 3 % methanol (pH 4.2). Separation of 5HT and 3-methoxytyramine (3-MT) was confirmed prior to each dialysis experiment. Compounds were detected with an LC-4B amperometric detector (Bioanalytical Systems, West Lafayette, IN) with a glassy carbon working electrode maintained at a potential of +0.7 V relative to an Ag/AgCl reference electrode. Data were recorded using a Hewlett Packard Integrator.

Striatal Dopamine and Serotonin Content in Tissue

MDMA, METH and/or MAL were locally perfused into the striatum. Only tissue directly surrounding the probe was assayed for neurotransmitter content. We have observed previously that damage produced by the probe itself may affect neurotransmitter content (unpublished observation). Therefore, in all studies we compared tissue surrounding the probe perfused with drug to the contralateral side perfused with vehicle perfusion medium.

One week following dialysis, rats were killed by rapid decapitation and the brain quickly removed and frozen with dry ice. Forty μm thick coronal sections were taken until both probe tracts were visualized. A 400 μm thick section was then collected for the dissection of tissue around the probe tracts. The tissue approximately 0.5 mm to either side of the tract was dissected out using a dissecting microscope (40x) and stored at -85°C until analysis.

Tissue was sonicated in 300 μl of 0.1 M perchloric acid, centrifuged at 14,000x g for 6 min. Dopamine and 5HT were separated and quantified using HPLC with ECD as described above. Concentrations were expressed as nanogram per milligram protein. Protein content was determined using a Bradford Protein Assay.

Statistics

Dopamine and 5HT tissue concentrations from striata perfused with drug were compared to those striata perfused with vehicle and were analyzed by 1-way analysis of variance (ANOVA) followed by Newman-Keuls where appropriate. Extracellular dopamine was analyzed with a 2-factor repeated measures ANOVA across time and Newman-Keuls where appropriate. Comparisons between dopamine and 5HT release with MDMA and METH groups in the dialysis experiments were done by area under the curve (AUC) and analyzed using a Students t-test. For all analysis, significance was set as $\alpha = 0.05$.

RESULTS

Acute Effects

The local perfusion of MDMA, MAL or the combination of MDMA/MAL, produced a significant increase in extracellular dopamine concentrations (2-way repeated measures ANOVA, $F_{27,306} = 342.2$, $p < 0.001$). During the perfusion of MDMA, dopamine concentrations peaked 1 hour after the perfusion began and remained elevated throughout the 8-hr period (Fig 1A). The local perfusion of 100 mM MAL also caused an initial significant increase in extracellular dopamine but the concentrations returned to baseline levels by the 3rd hour of drug perfusion (Fig 1A). In contrast to MDMA alone, the increase observed with MDMA/MAL was not maintained and by the 3rd hour of the perfusion, extracellular concentrations were not different from the Dulbecco's Vehicle group (Fig 1A).

The local perfusion of METH, or the combination of METH/MAL also increased striatal extracellular dopamine during the 8-hr perfusion period (2- way repeated measures ANOVA, $F_{27,247} = 39.8$, $p < 0.001$). Dopamine concentrations were maintained with the perfusion of METH alone. The combination of METH and malonate (METH/MAL) also produced a rapid significant increase that was not sustained and eventually returned to baseline values.

The perfusion of METH produced a greater increase in dopamine release compared to MDMA (AUC, Students t-test, df= 11, t=3.529, p= 0.005). The increase observed with the combination of METH/MAL was not different from that observed for MDMA/MAL (AUC, Student's t-test, df = 10, t = 0.775, p = .225).

Extracellular 5HT concentrations increased significantly during the perfusion of MDMA MAL, or MDMA/MAL (2-way repeated measures ANOVA, $F_{27,216} = 6.91$, $p<0.001$). The initial increase caused by MDMA or MAL was similar during the first hour of the perfusion and

persisted throughout the perfusion (Fig 2A). The increase observed with MDMA/MAL was greater than MDMA or MAL alone.

Extracellular 5HT concentrations were also increased during the local perfusion of METH, or METH/MAL (2-way repeated measures ANOVA, $F_{27,198} = 8.89$, $p < 0.001$). The perfusion of METH caused an initial increase in 5-HT that was similar in magnitude to that observed by MDMA; however this increase was not maintained and 5HT concentrations returned to baseline by the 5th hour of the perfusion with METH (Fig 2B). The peak increase in extracellular 5HT with METH/MAL was greater than METH or MAL alone. Although the combination of METH/MAL caused a pattern of release similar to MDMA/MAL, the increase in 5HT release was greater with MDMA/MAL (AUC, Student's t-test, df=12, $t = 2.42$, $p = 0.016$).

There were no significant changes in rectal body temperatures during the perfusion of MDMA, METH, MAL or the combination of MAL with either drug (Fig 3).

Tissue Concentrations of Dopamine and Serotonin

The local perfusion of MDMA or METH alone did not change striatal dopamine tissue content measured 7 days after the perfusion (Fig 4A,B). The local perfusion of MAL did however, decrease DA tissue content compared to Dulbecco's controls (ANOVA, interaction $F_{3,66} = 9.497$, $p< 0.001$). The perfusion of MDMA/MAL depleted DA concentrations to a greater extent than that observed with MAL alone (ANOVA, interaction $F_{3,70} = 28.837$, $p <0.001$, Fig 4A). The combination of METH/MAL also depleted DA tissue content, but the magnitude of the depletion was not significantly different from that observed with MAL alone (Fig 4B).

Striatal 5HT tissue concentrations were unchanged by the local perfusion of MDMA, METH, MAL or the combination of METH/MAL (Fig 5A,B). Only the combination of

MDMA/MAL caused a significant reduction in 5HT tissue concentrations (ANOVA, interaction $F_{3,70} = 3.245$, $p < 0.001$, Fig 5A).

DISCUSSION

The acute and long-term effects of a local striatal perfusion with MDMA or METH and their interactions with the mitochondrial complex II inhibitor, MAL, were examined to investigate the differential toxicity between MDMA and METH. MDMA and METH both increased striatal 5HT and dopamine release during local perfusion but did not produce long-term depletions of these transmitters. MAL also acutely increased 5HT and dopamine release but only produced long-term depletions in dopamine tissue content. The combined perfusion of METH/MAL, as well as MDMA/MAL, exacerbated the increased release of 5HT and dopamine and produced a long-term depletion of dopamine content. Moreover, the effect of MAL was synergistic with MDMA on the depletion of 5HT and dopamine tissue content.

The present findings support the hypothesis that a compromised bio-energetic state underlies the toxic effects of MDMA and METH. Because hyperthermia disrupts cellular energetics, inhibits mitochondrial function,[10,11,26] and mediates in part, amphetamine toxicity,[6,21] the toxic effects following the systemic administration of MDMA and METH may be produced through a hyperthermia-dependent compromise in bio-energetic state. Conversely, the absence of toxicity following the local infusion of MDMA and METH may be due to the lack of effect on body temperature (Fig 3). Consequently, we combined the local infusion of either amphetamine with the inhibition of energy metabolism to examine the role of bio-energetic stress in mediating the toxicity typically observed following the systemic administration of these drugs. Because MAL inhibits succinate dehydrogenase and decreases striatal ATP,[1] the local infusion of MAL may reproduce some of the biochemical effects associated with hyperthermia. In fact, the effects of systemic MDMA and METH appear to parallel the consequences of MAL on energy production. Along these lines, MDMA increases glycogen breakdown in astroglial-rich cell

cultures,[31] METH decreases striatal ATP, and both MDMA and METH inhibit cytochrome c oxidase activity.[7] Thus, the similarities between mitochondrial inhibition and the effects produced by MDMA and METH suggest that MAL would interact with METH and MDMA to produce acute and long-term effects greater than those seen with MAL, MDMA or METH alone.

Acute Effects

METH increased striatal dopamine release more than MDMA (Fig 1). In contrast, 5HT was increased more by MDMA than by METH (Fig 2). Although the peak increase in 5HT release was similar, 5HT remained significantly elevated during MDMA perfusion. In contrast, 5HT returned toward basal values before the termination of METH perfusion. These results are consistent with previous findings that MDMA is a more potent releaser of 5HT.[5] Moreover, this is the first in vivo evidence that METH, compared to MDMA, is a more potent releaser of dopamine.

The perfusion of MAL significantly increased the extracellular concentrations of dopamine and 5HT. Although the mechanism is unknown, the transient increase, despite the continuous perfusion of the drug alone or in combination with the amphetamines, suggests that transmitter efflux produced by MAL alone, or in combination with either MDMA or METH, is not sustained during mitochondrial inhibition. Although speculative, perhaps this is due to depleted levels of energy stores by malonate that are insufficient to sustain the continued reversal of the dopamine transporter by the perfusion of the amphetamines.

The combination of MDMA or METH with MAL enhanced the acute effects of the amphetamines on dopamine and 5HT release. Both MDMA/MAL and METH/MAL produced a synergistic increase in dopamine release (Fig 1) as revealed by an increase greater than the sum

of the individual effects of the drugs. A MAL-induced increase in intracellular calcium or the accumulation of intracellular sodium resulting from the inhibition of Na/K ATPase may have augmented the MDMA and METH-induced transporter mediated DA release. In contrast to the effects on dopamine, only an additive effect on 5HT release was observed with METH/MAL, whereas a synergistic increase was observed with the combination of MDMA/MAL (Fig 2). Similar to the previously identified long term toxic effects of MAL on DA neurons,[4,45] it is possible that DA neurons also are more sensitive to the interaction between the acute pharmacological effects of MAL and the amphetamines. In addition, it appears that the combination of MAL and amphetamine releases dopamine and 5HT by different undefined mechanisms.

Long-term Effects

The present results confirm previous findings that the central administration of MDMA[28] or METH[7] does not produce long-term depletions of dopamine or 5HT, respectively. The lack of neurotoxicity is interesting in light of the findings that dopamine, 5HT and/or glutamate release appear to mediate the toxic effects of these drugs following systemic administration.[24,25,35,36,39] Although elevated extracellular concentrations of dopamine and 5HT were observed following the local perfusion of MDMA and METH, this route of administration did not produce long-term depletions of striatal 5HT and dopamine. These data are nevertheless consistent with the finding that extracellular dopamine is not associated with the long-term toxic effects of METH.[20] In contrast to 5HT and dopamine release, we have demonstrated previously[7] that glutamate release is not elevated using an identical treatment regimen and route of administration, i.e. 8 hrs continuous local perfusion of METH. Therefore, the absence of an increase in glutamate may

account for the absence of toxicity following the local perfusion of METH. Regardless, this mechanism does not explain why the local/central administration of MDMA does not deplete tissue 5HT[28] (Fig 2B) since the systemic administration of toxic doses of MDMA also does not increase glutamate release.

The observed MAL-induced decrease in dopamine tissue content might be a consequence of increased glutamate release[7,22] and the subsequent enhancement of the increase in intracellular calcium produced by the inhibition of mitochondrial oxidative phosphorylation.[16] The finding that MAL depleted tissue dopamine but not 5HT content (Figs 4, 5) is consistent with previous studies showing that DA neurons are particularly vulnerable to the toxic effects of mitochondrial inhibition produced by MAL.[4,45]

The long-term effects of the perfusion of MDMA or METH alone or in combination with MAL differed in several interesting aspects. As noted above, MDMA and METH did not deplete dopamine or 5HT when measured 7 days after the perfusion. When METH was combined with MAL, no further dopamine depletion was observed beyond that produced by MAL alone. However, if a higher concentration of malonate (200 mM) is used with METH, a synergistic effect on the long-term depletion of dopamine is observed.[7] An interesting result of the present study was that the MDMA/MAL combination also produced a synergistic depletion of dopamine. This effect is not related to the acute effects on dopamine release since dopamine release was similar with METH/MAL and MDMA/MAL. The observed differential toxicity may be due to the massive release of 5HT associated with MDMA/MAL, resulting in the production of the toxic metabolite, tryptamine-4,5-dione.[14] Regardless, MAL appears to synergize with MDMA to deplete dopamine, an effect similar to that observed during the

systemic administration of METH or at higher concentrations of locally applied MAL in combination with METH.[7]

The effects on the 5HT system after MAL were limited to the combination with MDMA. Serotonin was not depleted with either METH or MAL alone or in combination. In contrast, MDMA/MAL produced a marked depletion of 5HT that was greater than the sum of the individual effects of the drugs. This is evidenced by the absence of a 5HT depletion with either drug alone compared to the significant depletion produced by MDMA/MAL. The enhancement of 5HT release may be related to these long-term changes in 5HT content. Stimulation of the 5-HT $_{2A}$ receptor appears to mediate the toxic effect of MDMA.[23,31,37] $5HT_{2A}$ receptor stimulation activates protein kinase C (PKC) through phosphotidylinositol 4,5-biphosphate,[9] and increases intracellular calcium.[18,19,29] Moreover, the binding of MDMA to the 5HT transporter also could produce translocation of PKC[19] and similarly increase intracellular calcium. These effects of MDMA on second messengers, in combination with the inhibition of mitochondrial function and the diminished sequestration of calcium that ensues,[2] could synergize and damage 5HT terminals. The differential effects of MDMA and METH in combination with MAL on 5HT terminals also could be explained by a calcium-dependent mechanism. It has been demonstrated that the calcium channel blocker, flunarizine, attenuated the decrease in tryptophan hydoxylase produced by MDMA but not METH,[15] suggesting that different mechanisms underlie the effects of these drugs on the 5HT system.

Overall, the present results highlight differences in the importance of energy balance to the function of DA and 5HT neurons and to the differential toxic effects of MDMA and METH. The local *in vivo* perfusion of MDMA and METH reproduced the classic acute effects of these drugs on dopamine and 5HT release but not the long-term effects on these neurons. The

inhibition of complex II by malonate preferentially damaged dopamine neurons compared to serotonin. The synergistic interaction between MAL and MDMA on dopamine concentrations suggests a compromised energetic state interacts with the toxic and pharmacologic effects of MDMA and parallels the profile observed with METH. Moreover, the interaction between the effects of MDMA on 5HT and MAL-induced inhibition of mitochondrial function emphasize the relative importance of the role of metabolic stress in the degeneration of 5HT compared to DA neurons.

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FIGURE LEGENDS

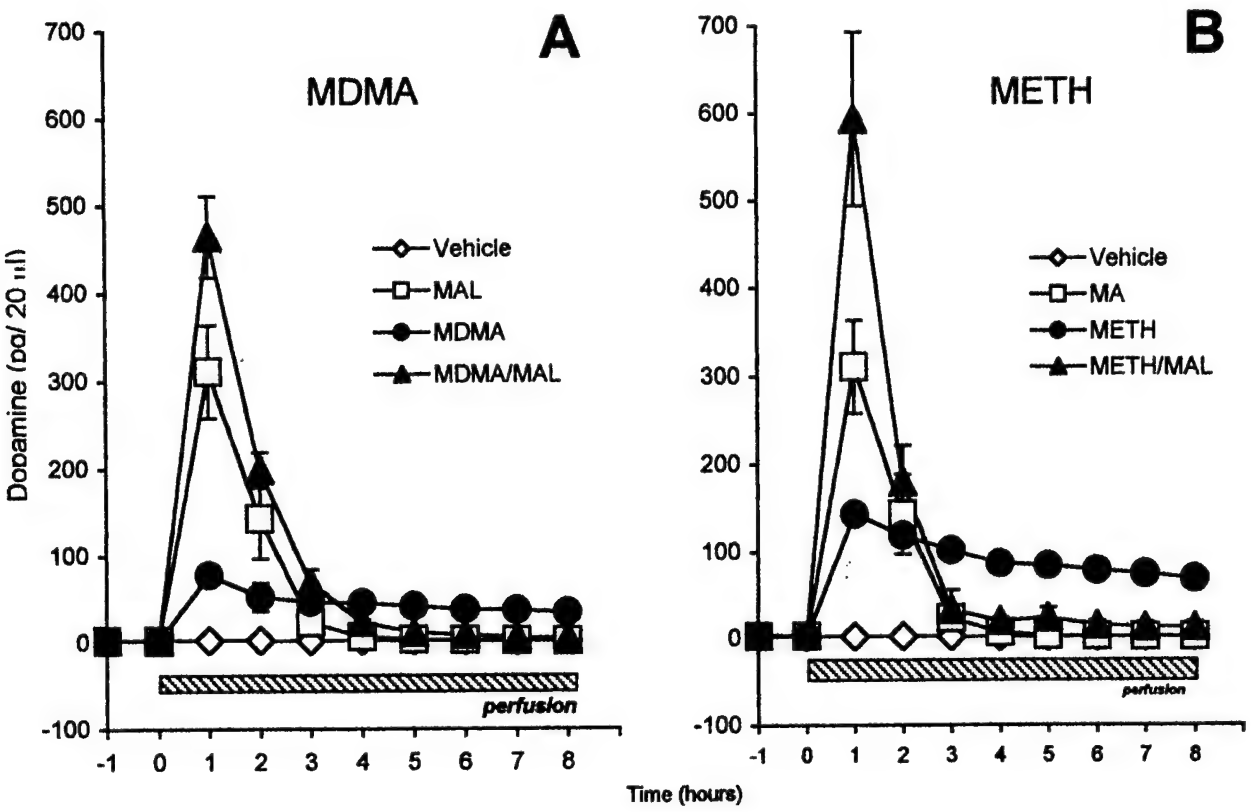
Figure 1. Extracellular dopamine in the striatum during the local perfusion of: **A)** Vehicle, MDMA (100 μM, n=7), MAL, or MDMA/MAL (n=9); **B)** Vehicle (n=13), METH (100 μM, n=6), MAL (100 mM, n=7) or METH/MAL (n=3); The local perfusion of METH increased extracellular dopamine concentrations more than that observed with MDMA (AUC, Students t-test, $p < 0.05$). Perfusion of malonate alone significantly increased extracellular dopamine but this increase was not sustained and dopamine concentrations returned to baseline by the 5th hour of drug perfusion. MAL in combination with METH produced a synergistic but transient increase in dopamine that was similar to the increase observed with MDMA/MAL (AUC, Students t-test, $p > 0.05$).

Figure 2. Extracellular concentrations of serotonin in the striatum during the reverse dialysis of: **A)** Vehicle, MDMA (100 μM, n=7), MAL, MDMA/MAL (n=9); **B)** Vehicle (n= 9), METH (100 μM, n= 7), MAL (100 mM, n=5), or METH/MAL (n=6); The local perfusion of MDMA, METH and MAL all significantly increased extracellular concentrations of 5HT. The combination of METH/MAL produced a significant increase in extracellular 5HT that was additive compared to METH and MAL alone. MAL/MDMA caused a synergistic increase in 5HT release that was greater than that observed with METH/MAL (AUC, Student's t-test, $p < 0.05$)

Figure 3. Effect of local perfusion of MDMA (100 μM, n=3), METH (100 μM, n=3), MAL (100 mM, n=6), or the combination of MDMA/MAL (n=6) or METH/MAL (n=4) into the striatum on core body temperature (°C). Body temperature was not changed by any of the drugs during the 8-hour perfusion.

Figure 4. Dopamine tissue content 7 days following perfusion with: **A)** Vehicle (n=36), MDMA (100 μM, n=7), MAL, or the combination of MDMA/MAL (n=20); **B)** Vehicle (n=33), METH (100 μM, n=10), MAL (100 mM, n=11) or the combination of MAL/METH (n=17). Concentrations of dopamine were unchanged in the MDMA and METH groups. The perfusion of malonate alone resulted in a significant reduction in dopamine content compared to Vehicle (*$p<0.05$). The combination of MAL/METH was not significantly different than malonate alone. In contrast, the combination of MAL/MDMA produced a greater decrease in dopamine compared to MAL ($p<0.05$).

Figure 5. 5HT tissue content 7 days following perfusion with **A)** Vehicle (n=36), MDMA (100 μM, n=7), MAL, or the combination of MDMA/MAL (n=20); **B)** Vehicle (n=33), METH (100 μM, n=10), MAL (100 mM, n=11) or the combination of MAL/METH (n=17). Serotonin tissue content was unchanged following perfusion with either MDMA or METH. The perfusion of malonate alone or the combination of MAL/METH also did not change 5HT tissue content. The 5HT content in the MAL/MDMA group was significantly less than the other groups (*$p<0.05$).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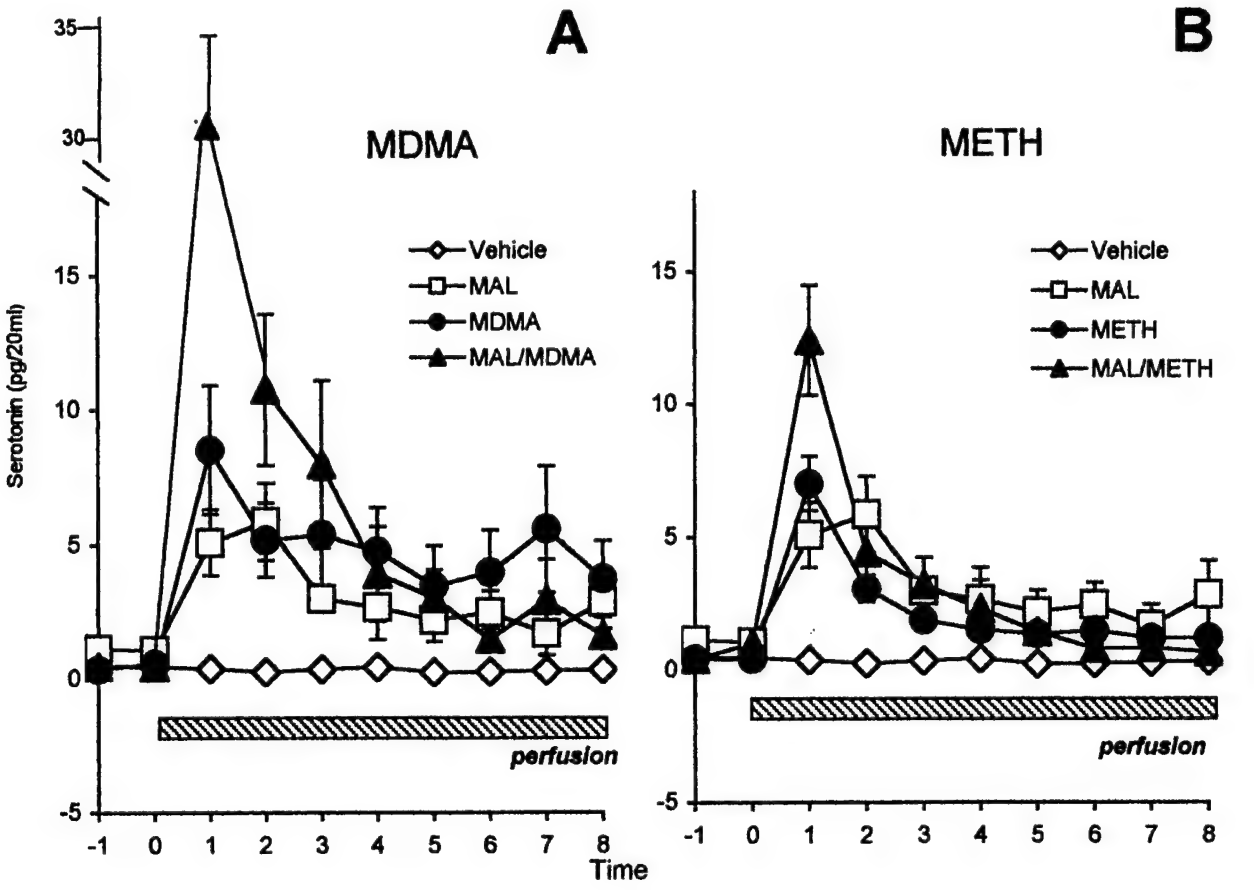
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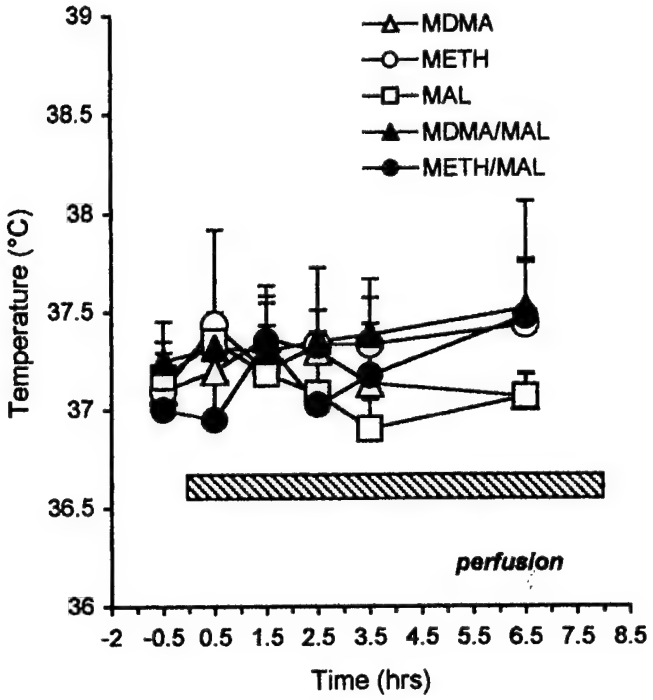
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perfusion
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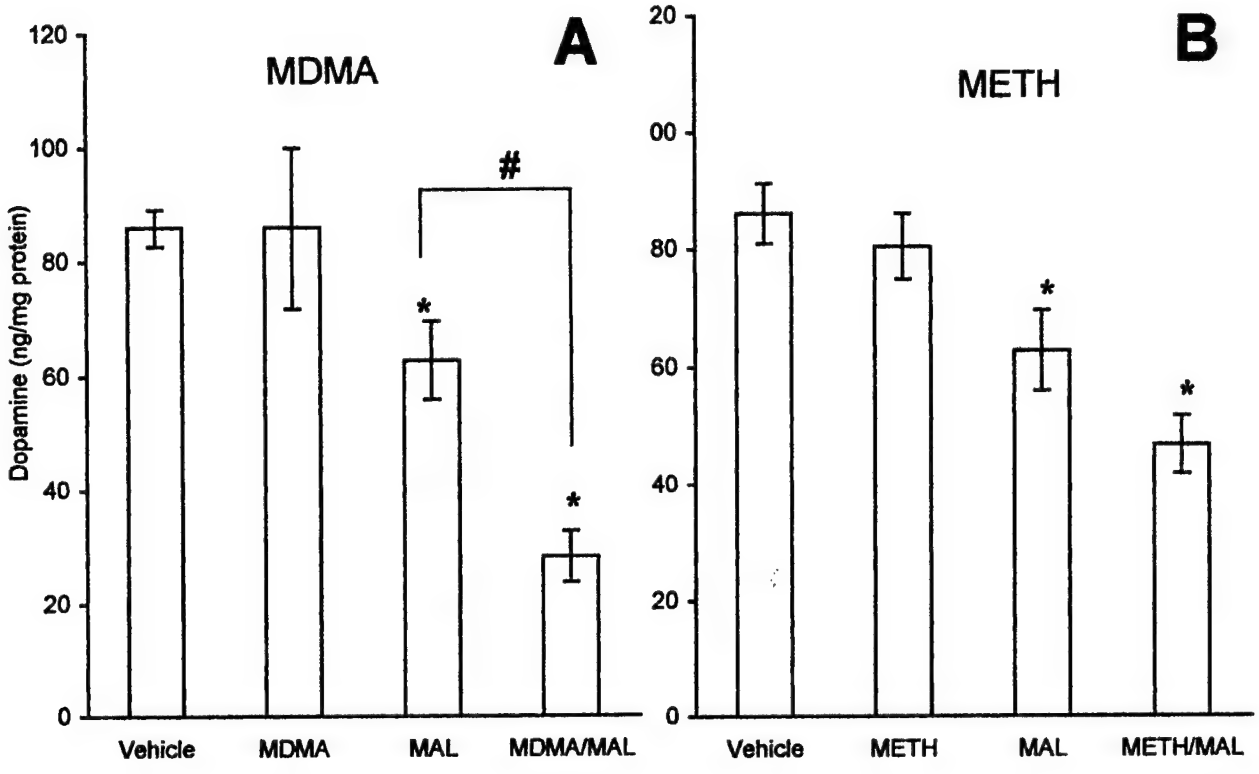
MDMA
A
METH
B
Dopamine (ng/mg protein)
120
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Vehicle
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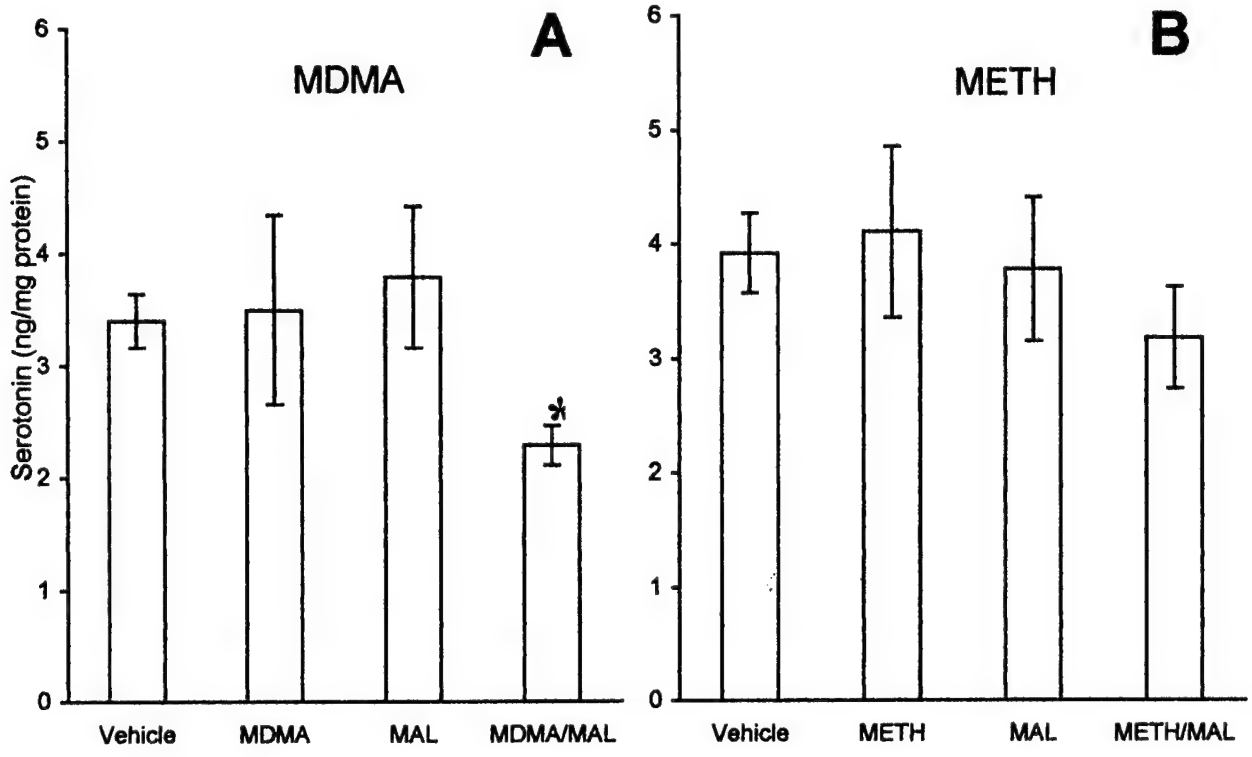
A
MDMA
Serotonin (ng/mg protein)
0
1
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Vehicle
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Short communication

Methamphetamine selectively alters brain glutathione

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Abstract

As methamphetamine-induced neurotoxicity has been proposed to involve oxidative stress, reduced and oxidized glutathione (GSH and GSSG, respectively), vitamin E and ascorbate were measured in the striata of rats killed 2 or 24 h after a neurotoxic regimen of methamphetamine. At 2 h, methamphetamine increased GSH and GSSG (32.5% and 43.7%, respectively) compared to controls at 2 h. No difference was seen in glutathione at 24 h, and in vitamin E and ascorbate at either time point. These findings indicate selectivity of methamphetamine for the glutathione system and a role for methamphetamine in inducing oxidative stress. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Methamphetamine; Glutathione; Oxidative stress

1. Introduction

Methamphetamine produces long-term depletion of dopamine and serotonin (5-hydroxytryptamine; 5-HT) tissue contents (Seiden et al., 1975–76), a reduction in tyrosine hydroxylase and tryptophan hydroxylase activity (Hotchkiss et al., 1979; Hotchkiss and Gibb, 1980), and decreases in the density of dopamine and 5-HT uptake sites (Wagner et al., 1980). While many long-lasting toxic effects have been demonstrated, the mechanisms underlying this toxicity have yet to be determined.

Recent studies indicate that oxidative stress and alterations in cellular metabolism mediate methamphetamine toxicity. Striatal ATP is reduced following high doses of methamphetamine (Chan et al., 1994). Moreover, methamphetamine increases the production of free radicals and produces oxidative damage (Yamamoto and Zhu, 1998), presumably via the excess release of dopamine and/or glutamate (Yamamoto et al., 1998). Additionally, the administration of antioxidants and spin trapping agents has been reported to protect against methamphetamine-induced toxicity (DeVito and Wagner, 1989; Cappon et al., 1996; Yamamoto and Zhu, 1998). Therefore, concentrations of endogenous antioxidants, such as glutathione, ascorbate, and vitamin E, may be altered as a consequence of methamphetamine-induced changes.

Moszczynska et al. (1998) demonstrated a modest but significant reduction in total glutathione in the brain following subchronic dosing of methamphetamine. However, it is unknown if the decrease in total glutathione is a consequence of increased oxidation. The purpose of the present study is to extend these findings to include parallel measurement of oxidized glutathione (GSSG) and reduced glutathione (GSH). We hypothesized that GSSG in the striatum will increase, while GSH will decrease after methamphetamine. We also compared the effects of methamphetamine on another water-soluble antioxidant, ascorbate, and compared it to these effects on vitamin E, a lipid soluble antioxidant.

2. Methods

2.1. Materials

Methamphetamine HCl, dinitrofluorobenzene, iodoacetic acid, glutathione ethyl ester, and GSSG were purchased from Sigma (St. Louis, MO).

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2.2. Drug treatment

Male Sprague–Dawley rats (190–350 g) were treated over an 8 h period at 2-h intervals with either methamphetamine (10 mg/kg) dissolved in 0.9% saline or saline alone. The rats were killed by decapitation at 2 or 24 h following the fourth injection. The striata were dissected, immediately frozen on dry ice, and stored at −80°C until assayed. All animal procedures were conducted in compliance with the NIH Guide for the Care and Use of Laboratory Animals, and were approved by the local Institutional Animal Care and Use Committee.

2.3. Biochemical assays

2.3.1. Glutathione analysis

Striata were analyzed by a modification of the method of Reed et al. (1980). The striatum was homogenized in 0.1N $HClO_4$ (800 μl) and centrifuged at 14 000 × *g* for 6 min at 4°C. The supernatant (100 μl) was incubated with 0.88 M iodoacetic acid (20 μl) and excess sodium bicarbonate (approximately 15 mg) for 1 h in the dark, at room temperature. The solution was incubated with 100 μl of 1.5% dinitrofluorobenzene (in methanol) for 4 h in the dark, at room temperature. Diethyl ether (500 μl) was then added and the solution was centrifuged for 20 min at 2000 × *g*. The aqueous layer was separated and stored at 0°C. Protein content was analyzed according to the method of Bradford.

The aqueous layer was analyzed by high pressure liquid chromatography (HPLC) with ultraviolet spectroscopy (λ = 355 nm). GSH and GSSG were separated chromatographically on a Luna 3 μ C18, 150 × 4.6 mm column (Phenomenex, Torrance, CA). The mobile phase (pH 3.5) consisted of 0.8 M sodium acetate trihydrate, 15% glacial acetic acid and 20% methanol. Column temperature was maintained at 33°C. The retention times of GSH and GSSG under isocratic conditions were 14 and 96 min, respectively.

2.3.2. Vitamin E assay

Vitamin E was measured by HPLC, coupled with electrochemical detection. The striatum was homogenized in ethyl acetate (600 μl) and centrifuged at 14 000 × *g* for 6 min. The supernatant (20 μl) was injected into a 4.6 × 250 mm Ultrasphere C18 reverse phase column (5 μm particle size; Beckman, San Ramon, CA). The mobile phase was 96% methanol: 4% ddH_2O and 40 mM sodium perchlorate.

2.3.3. Ascorbate assay

The striatum was homogenized in 1 ml of 0.1 N $HClO_4$ and centrifuged for 6 min at 14 000 × *g*. The supernatant (20 μl) was assayed for ascorbate by HPLC with electrochemical detection. The mobile phase (pH 4.5) was 21.7 mM sodium acetate, 2.5 nM tridecylamine, 3.3% glacial acetic acid and 6% methanol. Ascorbate was separated from biogenic amines and metabolites using a C18 reverse phase column (2 × 100 mm, 3 μm) (Phenomenex). The potential of the glassy carbon electrode was maintained at 0.6 V vs. an Ag/AgCl reference electrode.

3. Results

The total striatal GSH and GSSG content 2 h after the fourth injection of methamphetamine was increased by 33% ($P < 0.01$), and 44% ($P < 0.02$), respectively (Fig. 1). The GSH content was 273.7 ± 21.1 μg/mg protein in saline control rats and 362.8 ± 26.6 μg/mg protein in methamphetamine treated rats. The GSSG content was 10.1 ± 0.9 ng/mg protein in the saline group and 14.5 ± 1.4 ng/mg protein in the methamphetamine-treated rats. The total GSH in striatum of the rats killed 24 h following the last injection was not different in saline and in methamphetamine-treated rats (saline: 263.3 ± 21.6 μg/mg protein; methamphetamine: 276.2 ± 18.0 μg/mg protein; $P > 0.30$) (Fig. 1). Similarly, the total GSSG content in methamphetamine-treated rats and in the con-

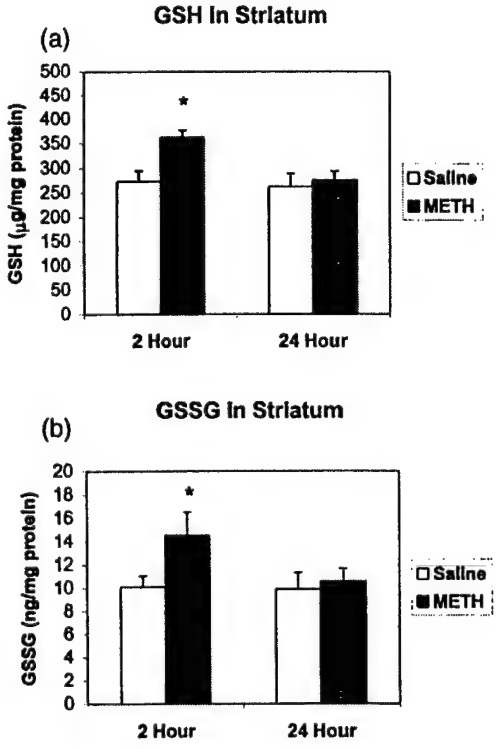

Fig. 1. (a) GSH ($n = 10$ for 2 and 24 h saline and 2 h methamphetamine; 13 for 24 h methamphetamine) and (b) GSSG ($n = 6$ for 2 h saline; 7 for 2 h methamphetamine and 24 h saline; and 9 for 24 h methamphetamine) in striatum 2 or 24 h after methamphetamine administration. Results are expressed as means ± S.E.M. Data was analyzed with a Student's *t*-test. * $P < 0.05$ compared to saline controls.

Table 1
Vitamin E and ascorbate in striatum following methamphetamine or saline administration. Results are shown as means ± S.E.M. ($n = 6$/group)

	Saline	Methamphetamine	
		2 (h)	24 (h)
Vitamin E (ng/mg wet tissue weight)	8.57 ± 0.34	9.02 ± 0.46	8.53 ± 0.40
Ascorbate (ng/μg protein)	5.66 ± 0.42	6.11 ± 0.37	5.76 ± 0.42

trols was not different (saline: 9.9 ± 1.9 ng/mg protein; methamphetamine: 10.6 ± 1.1 ng/mg protein; $P > 0.30$).

The total content of vitamin E was the same after methamphetamine as after saline administration (Table 1). The average total vitamin E content in control rat striatum was 8.5 ± 0.3 ng/mg wet tissue weight, and in the methamphetamine-treated rats, it was 9.0 ± 0.4 and 8.5 ± 0.4 ng/mg wet tissue weight after 2 and 24 h, respectively. Similarly, total ascorbate in the striatum of methamphetamine-treated animals was not different ($P > 0.05$) from that in the controls at either 2 or 24 h after the fourth injection (Table 1). The mean ascorbate content in control rats was 5.6 ± 0.4 ng/μg protein, and 6.1 ± 0.3 and 5.7 ± 0.4 ng/μg protein for the 2 and 24 h methamphetamine groups, respectively.

4. Discussion

Antioxidants in rat striatum were measured after the administration of methamphetamine. Total GSH and GSSG were both increased at 2 h following the fourth injection of methamphetamine, but returned to their control values at 24 h. No differences in vitamin E and ascorbate in striatum were observed at either 2 or 24 h after methamphetamine.

Methamphetamine produces oxidative stress in the striatum through the production of hydroxyl free radicals (Yamamoto and Zhu, 1998). GSH reacts non-enzymatically with hydroxyl radicals to produce GSSG (Griffith, 1999). The enzymatic formation of GSSG has also been shown to be a consequence of an oxidation (Han et al., 1999) and of a neuroprotective response to an oxidative stressor (Iwata-Ichikawa et al., 1999). However, Moszczynska et al. (1998) found that glutathione peroxidase and glutathione reductase activities were unaltered following repeated systemic administrations of methamphetamine. It follows that the increase in GSSG at 2 h after methamphetamine could be the result of elevated accumulation due to the combination of increased non-enzymatic oxidation of GSH to GSSG and lack of an increase in enzymatic reduction. The alteration of GSSG in the presence of oxidative stress is evidence for the oxidative effects of methamphetamine on the glutathione system.

GSH was also elevated 2 h after the fourth injection of methamphetamine. This increase could have been due to an increased synthesis of GSH in glial cells. It has been shown that the rate-limiting enzyme in GSH synthesis, γ-glutamylcysteine synthetase, is up-regulated in glial cells, as well as in other organs, in the presence of oxidative stress (Iwata-Ichikawa et al., 1999; Moellering et al., 1998; Woods et al., 1999). Therefore, the increased synthesis of GSH may be responsible for its increase at 2 h following methamphetamine. By 24 h after methamphetamine, however, the activity of the glutathione system has returned to its control values (Fig. 1). An alternative, but less likely explanation, is that the increase in glutamate caused by methamphetamine administration (Nash and Yamamoto, 1992) disrupts the feedback inhibition by GSH on γ-glutamylcysteine synthetase (Richman and Meister, 1975), an effect observed in the rat kidney, but yet to be observed in the brain.

The increase in GSH after methamphetamine contrasts with the decrease in GSH observed earlier (Moszczynska et al., 1998). The twofold increase in the total dose of methamphetamine used in the Moszczynska et al (1998) study could have produced greater oxidative stress and, consequently, long-term depletion of total glutathione stores. In contrast, the oxidative stress produced with the neurotoxic doses of methamphetamine in the present study may not have been sufficient to saturate the glutathione-related enzyme systems to produce measurable depletions of GSH in tissue. The acute increase in both GSH and GSSG in the striatum, however, supports the conclusion that methamphetamine alters the glutathione system, presumably in response to oxidative stress.

The present study also extended the examination of the effects of methamphetamine to include its effects on other antioxidants. Vitamin E is lipid soluble and primarily prevents free radical-induced lipid peroxidation (Chow, 1991). Vitamin E requires ascorbate and glutathione as co-factors to prevent lipid peroxidation and depends on ascorbate to reduce the oxidized vitamin E (Chow, 1991; Cardoso et al., 1998). Consequently, greater changes in glutathione and ascorbate concentrations may be necessary before vitamin E content is affected.

Ascorbate content also appeared unaffected by methamphetamine. Ascorbate functions as both a pro-oxidant and antioxidant. As a pro-oxidant, ascorbate reacts with iron to generate reactive oxygen species and produce oxidative damage (Chow, 1991), the effects of which can be reversed by GSH (Burk, 1982). In contrast, the antioxidant activity of ascorbate is mediated through the reduction of oxidized vitamin E and water-soluble oxidants (Chow, 1991). Therefore, because of these opposing actions, changes in total brain ascorbate concentrations may be difficult to detect after methamphetamine. Future studies differentiating between reduced and oxidized forms of ascorbate after methamphetamine should permit a clearer interpretation of the role of ascorbate in methamphetamine toxicity.

In conclusion, methamphetamine selectively affects the glutathione antioxidant system without influencing ascor-

bate and vitamin E. The sensitivity of glutathione to methamphetamine may be due to its diversified functions as a primary antioxidant and as a co-factor in the actions of other antioxidants. Regardless, alterations in total glutathione in the striatum provide further evidence that methamphetamine produces oxidative stress.

5. Uncited reference

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Acknowledgements

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APPENDIX 5

Abstracts

Roles for Metabolic and Oxidative Stress in Amphetamine Neurotoxicity

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Case Western Reserve University

Methamphetamine and MDMA ("Ecstasy") have acute and long-term effects on brain dopamine (DA) and/or serotonin (5HT) systems. The long-term changes are thought to be indicative of neurotoxicity and are manifested as decreases in the tissue content of DA and 5HT, decreases in the activities of the biosynthetic enzyme and biogenic amine transporters, as well as increases in histochemical markers such as silver staining and GFAP ***(see other abstracts)***. However, the exact mechanisms underlying these changes have not been elucidated.

Our laboratory has examined several possible processes that may be involved in mediating the long-term effects of the substituted phenylethylamines. These include the roles of glutamate, oxidative stress, bioenergetic compromise, and environmental stress. Our overall working hypothesis is that each of these mechanisms are essential but alone are insufficient to produce damage to DA and 5HT neurons. However, these components can synergize and together, result in long-term changes in DA and 5HT neurotransmission (4).

The results from current studies in this laboratory have focused on *in vivo* and *ex vivo* studies of the striatum. Our findings indicate that methamphetamine increases corticostriatal glutamate release (1) which, in turn, results in an increase in excitoxicity as evidenced by spectrin proteolysis. Furthermore, both methamphetamine and MDMA acutely increase the production of hydroxyl free radicals and the attendant increases in lipid and/or protein oxidation (2, 5). Conversely, the long-term depletions of DA are attenuated by antioxidants administered either locally or systemically (5).

In parallel with the oxidative stress and excitotoxic effects of methamphetamine and MDMA, these drugs also alter striatal bioenergetic state as revealed by the increased production of lactate and a transient inhibition of complex IV (cytochrome oxidase) of the mitochondria. Consistent with these findings, the local perfusion of substrates for complexes I and II for 6 hours after the systemic administration of methamphetamine attenuates the long-term depletion of striatal DA content (3). In addition, although the local and continuous perfusion of methamphetamine or MDMA does not increase glutamate release and has no long-term effect on DA or 5HT content, the local striatal perfusion of methamphetamine or MDMA synergizes with the inhibition of complexes I or II to produce a long-term depletion of DA and/or 5HT. Thus, methamphetamine and MDMA appear to acutely increase energy metabolism and eventually lead to a depleted bioenergetic state. Moreover, the long-term neurotoxic effects resulting from this energy compromise however, can be attenuated by the provision of metabolic substrates following the exposure to these drugs.

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***Neurotoxicity of Amphetamines and Related Stimulants*,**
Washington D.C., 1999

We also examined the long-term physiological and functional impact of MDMA administration. Unlike normal control rats that responded to restraint stress with increases in DA, 5HT, and glutamate release in the cortex, striatum or hippocampus, rats pretreated with MDMA do not respond with increases in these transmitters during a restraint episode. Therefore, rats treated with MDMA exhibit abnormal stress responses.

Overall, our results are supportive of the hypothesis that an increase in corticostriatal glutamate release synergizes with compromised metabolic and oxidative stress states to destroy a DA and/or 5HT axon terminal that has been stimulated continually by methamphetamine or MDMA. Future studies will be directed at the mechanistic underpinnings mediating the oxidative and metabolic stress effects of methamphetamine and MDMA.

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Neurotoxicity of Amphetamines and Related Stimulants, Washington D.C., 1999

Synergy of Methamphetamine with a Complex I Inhibitor Reduces Striatal Serotonin Content

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The neurotoxic effects of systemic methamphetamine (METH) administration include long term depletions of striatal dopamine and serotonin content. This effect is mediated by increased release of both dopamine and glutamate, which might lead to increased oxidative and/or metabolic stress and eventual death of neuronal terminals. Our studies have shown that local perfusion of METH into the striatum acutely increases extracellular dopamine, but does not result in the long-term depletion of either dopamine or serotonin tissue content. Presently, we have examined the interaction of a local perfusion of METH with rotenone, an inhibitor of mitochondrial respiration at Complex I, in order examine the role of metabolic stress in METH toxicity. METH (100 μM), rotenone (100 μM) or the combination of METH/rotonone (METH/ROT) was reverse dialysed into one side of the striatum for 8 hours while the opposite striatum was perfused with Dulbecco's saline. Extracellular concentrations of dopamine were monitored throughout the perfusion. One week following perfusions, samples of tissue immediately surrounding the probe site were dissected and assayed for dopamine and serotonin content. Perfusion of METH alone acutely increased extracellular dopamine by 30-fold; however, the tissue content of dopamine was unaltered when measured 7 days after the local perfusion of METH. Although perfusion of either rotenone alone or METH/ROT also increased extracellular dopamine (increased 8- and 70-fold respectively), dopamine tissue content one week later was unchanged. Serotonin tissue content was not altered by perfusion of either METH or rotenone alone but was decreased following METH/ROT. These results suggest that the local perfusion of METH or rotenone alone at the concentrations used is not toxic to dopamine or serotonin terminals in the striatum. In contrast, METH synergizes with rotenone to selectively damage serotonin terminals within the striatum.

8th International Conference on In Vivo Methods, Stony Brook, NY, 1999.

The Interaction Between the Reverse Dialysis of Methamphetamine and an Inhibitor of Complex I on the Depletion of Striatal Dopamine and Serotonin

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Introduction

The neurotoxic effects of systemic methamphetamine administration include long-term depletions of dopamine and serotonin tissue concentrations. Although the mechanisms of methamphetamine toxicity are not fully understood, it is thought that these effects are mediated by an increase in dopamine and glutamate release which leads to the eventual death of neuronal terminals [1]. This acute release of dopamine following METH administration occurs via the reversal of the dopamine transporter[2] and could cause an increase in energy consumption for maintenance of appropriate ion gradients. METH has, in fact, been shown to deplete striatal ATP concentrations[3], and substrates of energy metabolism attenuate METH toxicity suggesting that perturbations in energy metabolism contribute to the neuronal damage produced by METH [4].

There are few studies that have examined the effects of methamphetamine following a local infusion directly into the striatum. Our studies have shown that local perfusion of METH into the striatum acutely increases extracellular dopamine, but does not result in long-term depletions of either dopamine or serotonin tissue concentrations. The objective of the current study was to examine the interaction between methamphetamine and rotenone, an inhibitor of electron transport at complex I. Rotenone administered systemically has been shown to selectively damage striatal neurons and may specifically target dopaminergic neurons [5,6]. We hypothesized that rotenone would synergize with the local perfusion of METH to produce damage to striatal dopamine and serotonin terminals.

Materials and Methods

In vivo microdialysis was used to examine the interaction between methamphetamine and the complex I inhibitor, rotenone. METH (100 μM), rotenone (100μM) or the combination of METH/rotenone (METH/ROT) was reverse dialysed into one side of the striatum for 8 hours via a microdialysis probe, while the opposite striatum was perfused with Dulbecco's saline. Extracellular concentrations of dopamine and glutamate were monitored throughout the perfusion by HPLC/ECD. One week following perfusions, samples of tissue immediately surrounding the probe site were dissected and assayed for dopamine and serotonin content.

Results and Discussion

Perfusion of METH alone acutely increased dopamine release by 30-fold, while the combination of METH/ROT increased dopamine release 25-fold. No acute increase in dopamine release was observed following perfusion with rotenone alone.

Table 1.Effect of local drug perfusion on dopamine efflux in striatum(area under the curve)

	Dulbeccos	METH	Rotenone	METH/ROT
AUC±SEM	5.5±4.2	90.2±28.4	8.42±10.4	56.7±33.1

Extracellular glutamate concentrations were similar in all groups and remained stable throughout the perfusion. Dopamine tissue concentrations, measured 7 days after the perfusion, were not altered by METH, rotenone or the combination of METH/ROT. Serotonin tissue content was unchanged following perfusion with METH and rotenone alone, but was decreased following METH/ROT.

Table 2. Striatal DA and 5-HT content (ug/pg protein) 7 days following drug perfusion

	Dulbeccos	METH	Rotenone	METH/ROT
Dopamine	165±15.2	128±14.3	140±10.0	139±24.1
Serotonin	4.9±0.5	6.1±1.0	6.2±1.4	3.4±0.3*

*$p<0.05$, different from all other drug groups

These results suggest that the local perfusion of METH or rotenone alone, at the concentrations used, is not toxic to dopamine or serotonin terminals. In contrast, it appears that METH synergizes with rotenone to selectively damage serotonin terminals within the striatum. This selective serotonergic toxicity does not appear to be related to the acute release of dopamine or glutamate. Ongoing studies will examine extracellular serotonin release in response to the perfusion of METH, rotenone or METH/ROT.

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ACUTE AND LONG-TERM EFFECTS OF INTRASTRIATAL PERFUSIONS OF METHAMPHETAMINE OR MDMA. W.L. Nixdorf,[1*] K.B. Burrows,[1] G.A. Gudelsky,[2] and B.K. Yamamoto[1]. [1]Department of Psychiatry, Case Western Reserve Univ., Cleveland, OH 44106; [2]College of Pharmacy, Univ. of Cincinnati. The systemic effects of methamphetamine (METH) or 3,4-methylenedioxymethamphetamine (MDMA) include acute increases in extracellular dopamine (DA) and long term depletions of striatal DA and/or serotonin (5-HT) content. Few studies have examined in vivo, the effects of a local perfusion of METH or MDMA on the acute and long-term neurotoxic effects on DA and 5-HT. We have examined the effects of a local perfusion of METH or MDMA on DA and 5-HT release in the striatum. We also hypothesized that inhibition of complex II of the mitochondrion by malonate (MAL) would exacerbate the long-term depletion of DA and/or 5-HT tissue content produced by METH or MDMA. METH (100 μM) or MDMA (100 μM) was reverse dialysed into the striatum for 8 hr. In other experiments MAL (100 mM) alone or in combination with METH or MDMA was perfused into the striatum for 8 hr. One week later, tissue immediately surrounding the probe was assayed for DA and 5-HT content. Perfusion of METH or MDMA increased DA release (56-, 31-fold respectively). 5-HT release was increased 17-fold by METH but returned to baseline within 2 hours. MDMA increased 5-HT 32-fold but this increase was maintained throughout the perfusion. The perfusion of METH or MDMA alone did not change DA or 5-HT tissue content when measured 7 days later. MAL alone depleted DA, but not 5-HT. METH/MAL did not enhance the effects of MAL on DA or affect 5-HT. However, co-perfusion of MDMA with MAL enhanced the MAL-induced DA depletions and interacted synergistically to deplete 5-HT content. These results suggest that in vivo perfusion of both METH and MDMA acutely increase DA and 5-HT release but do not cause long-term depletions. It appears that a compromised metabolic state may be important to the long-term toxicity of MDMA.

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SYSTEMIC ADMINISTRATION OF METH OR MDMA INCREASES 3-NITROTYROSINE IN THE RAT STRIATUM

W.L. Nixdorf,[1*] G.A. Gudelsky,[2] and B.K. Yamamoto[1].

[1]Department of Psychiatry, Case Western Reserve University, Cleveland, OH 44106; [2]College of Pharmacy, University of Cincinnati, Cincinnati, OH 45267

Methamphetamine (METH) and 3,4-methylenedioxymethamphetamine (MDMA) are psychostimulants that produce long-lasting damage to dopamine and/or serotonin terminals in the striatum. Although the mechanisms responsible for this neurotoxicity are unclear, reactive nitrogen species and peroxynitrite in particular, might play a role in the cascade of events that leads to the destruction of neuronal terminals. Peroxynitrite oxidizes DNA, lipids, and proteins, and nitrates tyrosine to form 3-nitrotyrosine (3-NTY). We examined the effect of the systemic administration of METH or MDMA on 3-NTY 24 hours after drug administration using HPLC with electrochemical detection. Rats received 4 injections of METH, MDMA (10mg/kg) or saline every 2 hours and were killed 24 hours after the first injection. The striatum was removed and acid hydrolyzed prior to analysis with HPLC-ECD. 3-NTY was significantly increased 1.5-fold following METH and 3-fold following MDMA compared to saline controls. Although increases in peroxynitrite following METH might be due to enhanced glutamate release and the subsequent production of nitric oxide, the systemic administration of MDMA does not increase extracellular glutamate. Therefore, non-glutamatergic mechanisms may mediate the increase in peroxynitrite following MDMA. Overall, these results support the conclusion that protein oxidation contributes to METH and MDMA-induced toxicity. Ongoing studies will utilize western immunoblots to measure and confirm the increase in 3-NTY after METH and MDMA.

Supported by DA07606, DA07427, and DAMD 17-99-1-9479.

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Theme 1: J. Disorders of the Nervous System and Aging

Topic 1: 144. Drugs of abuse: amphetamines

Theme 2: J. Disorders of the Nervous System and Aging

Topic 2: 141. Neurotoxicity

Abstract Title: **LOCAL STRIATAL PERFUSION OF METHAMPHETAMINE SYNERGIZES WITH HYPERTHERMIA TO PRODUCE TOXICITY**

Contributing Authors:
1. K.B. Burrows[1*]
2. B.K. Yamamoto[1]

Institutions: 1. Dept Psychiatry, Case Western Reserve Univ, Cleveland, OH, USA

Key words: Microdialysis, Dopamine, Basal Ganglia, Neurotoxicity

Abstract: Systemic administration of methamphetamine (MA) depletes striatal dopamine (DA) and 5-HT content. In contrast, intrastriatal perfusion of MA does not deplete monoamines. Increased glutamate (GLU) release and hyperthermia mediate the toxicity of systemic MA. These studies examined the ability of local MA to interact with GLU and hyperthermia to deplete striatal DA and 5-HT content. MA (100μM) alone or in combination with GLU (100μM) was reverse-dialysed into one side of the striatum for 8 hr via a microdialysis probe. The other side was perfused with a vehicle or GLU alone. Rats were treated at room temperature (22°C) or in a warm environment (28-38°C) that increased body temperature to the degree obtained after systemic MA (39-41°C). One week later, tissue adjacent to the probe site was assayed for DA and 5-HT content. The continuous local perfusion of MA at room temperature produced a sustained increase in DA release (30 fold) but did not deplete DA content. At room temperature, perfusion of GLU in combination with MA enhanced DA release compared to MA alone, but did not affect DA tissue content. In a warm environment, perfusion of MA alone enhanced DA release and significantly depleted DA content by 30% ($p<0.01$). Co-perfusion of GLU and MA during hyperthermia further increased DA release but did not enhance DA depletions. Local perfusion of GLU or vehicle at either temperature did not alter DA release or content. 5-HT tissue content was not affected

by any treatment. Although local GLU perfusion enhanced DA release, the presence of hyperthermia was the determining factor mediating selective toxicity to DA terminals following local MA perfusion.

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ROLE OF METABOLIC INHIBITION IN METHAMPHETAMINE AND MDMA TOXICITY: EVIDENCE FOR DECREASED MITOCHONDRIAL FUNCTION FOLLOWING DRUG ADMINISTRATION. K. B. Burrows[1]*, G. A. Gudelsky[2], and B. K. Yamamoto[1], Dept. of Psychiatry[1], Case Western Reserve Univ., Cleveland, OH 44106, and College of Pharmacy[2], Univ. of Cincinnati, Cincinnati, OH 45267.

Methamphetamine (METH) and 3,4-methylenedioxymethamphetamine (MDMA), are potent psychostimulants known to damage dopamine and/or serotonin containing nerve terminals within the striatum. The exact mechanism of action has yet to be determined. Albers et al. (1996) demonstrated that infusion of the mitochondrial inhibitor, malonate, into the striatum increased dopamine depletion caused by systemic METH. Since METH also appears to enhance brain energy metabolism and decrease ATP stores, it is possible that perturbations of energy metabolism may also be involved in mediating the neurotoxic effects of these drugs. The **objectives** of this study were to: **(1)** Determine the disruption of energy metabolism following systemic METH (10 mg/kg x 4) or MDMA (10 mg/kg x 4) administration by examining histochemical staining for complex IV, cytochrome c oxidase (COX), of the electron transport chain. **(2)** Determine the time course of changes in COX activity by examining tissue 2 hrs, 24 hrs, or 7 days following drug administration. There is a rapid decrease in COX staining in the caudate/putamen (12-14%) and nucleus accumbens (16-17%) and substantia nigra (15-20%) following both METH and MDMA administration. This decrease in COX activity is transient and returns to control levels within 7 days post-drug, further suggesting that perturbations of the mitochondrial electron transport chain may mediate the neurotoxic effects of METH and MDMA.

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Intrastriatal perfusion of Methamphetamine and Interactions with Metabolic Stress: Inhibition of Complex II

Kristan B. Burrows, Wendy Nixdorf, and Bryan K. Yamamoto, Dept. of Psychiatry, Case Western Reserve University, Cleveland OH 44106

The systemic administration of methamphetamine (METH) depletes both dopamine (DA) and serotonin (5HT) content in the striatum. The neurotoxic effects of METH have been shown to be mediated by both increased extracellular concentrations of DA and glutamate, and may be due to excess oxidative and/or metabolic stress, which lead to terminal death. No studies to date have directly examined, *in vivo*, the effect of local intrastriatal perfusion of METH on DA and glutamate release in relation to long term changes in striatal neurotransmitter content. Moreover, the interaction between inhibition of energy metabolism and the direct perfusion of METH on the long-term decreases in DA and 5HT content has not been investigated.

METH (100 μM) was reverse-dialysed into one side of the striatum for 8 hours via a microdialysis probe. The other side was perfused with a Dulbecco's saline medium. Extracellular concentrations of DA and glutamate were monitored simultaneously throughout the perfusion. In other experiments, the succinate dehydrogenase inhibitor, malonate, alone or in combination with METH, was perfused on one side of the striatum while the other side was perfused with normal Dulbecco's. One week later, the tissue immediately adjacent to the probe site was assayed for DA and 5HT content. The continuous local perfusion of METH alone increased DA release by 30 fold, similar to that seen following systemic administration of toxic doses, but did not increase glutamate and did not deplete DA or 5HT content. Malonate perfusion increased both DA and glutamate overflow, and dose dependently decreased DA content. 5HT content was not as affected by malonate perfusions (200 mM malonate depleted DA by 60% and 5HT by only 30%). When METH was co-perfused with 200 mM malonate, striatal DA content was reduced by 75% and to a greater extent than on the side perfused with malonate alone. Co-perfusion of METH and 200 mM malonate did not enhance 5HT loss. This study demonstrates that increased extracellular DA alone in the absence of glutamate is not sufficient to cause long-term depletion of DA content after METH. Overall, the direct local perfusion of METH alone is not toxic to striatal DA terminals but synergizes with the local inhibition of energy metabolism to deplete DA content. Moreover, the inhibition of energy metabolism and the synergy with METH is selectively toxic to DA but not 5HT neurons.

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Intrastriatal perfusion of methamphetamine and interactions with metabolic stress: inhibition of Complex II

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Department of Psychiatry, Case Western Reserve University, Cleveland OH 44106

Introduction

Methamphetamine (METH) is a major drug of abuse with neurotoxic side effects which include a long-term depletion of striatal dopamine (DA) and serotonin (5-HT) content, and a decrease in the rate limiting enzymes and transporters for these neurotransmitter systems. The mechanisms mediating the neurotoxicity of METH remain unknown. These neurotoxic effects appear to be mediated by the increased release of dopamine and glutamate with a subsequent increase in hydroxyl free radicals[1,2]. Albers et al.[3] demonstrated that infusion of the mitochondrial inhibitor, malonate, into the striatum increased DA depletion caused by systemic METH. Since METH also appears to enhance brain energy metabolism[4] and decrease ATP stores[5], it is possible that perturbations of energy metabolism may also be involved in mediating the neurotoxic effects of METH.

Nearly all studies conducted to date have employed systemic administrations of METH, and thus any specific interpretations of the mechanisms mediating the neurotoxic effects of this drug are confounded by METH's potent peripheral sympathetic effects. No studies to date have directly examined, *in vivo,* the effect of local *intrastriatal* perfusion of METH on DA and glutamate release in relation to long term changes in striatal neurotransmitter content. Moreover, the interaction between inhibition of energy metabolism and the direct perfusion of METH on the long-term decreases in DA and 5-HT content has not been investigated. The objectives of the present study were to: (1) examine the neurotoxic effects of METH following the direct perfusion into the brain, and (2) test the hypothesis that a specific manipulation of energy metabolism, using the mitochondrial succinate dehydrogenase inhibitor, malonate, contributes to the long-term depletions of striatal DA and/or 5-HT content produced by psychostimulants.

Materials and Methods

In vivo microdialysis in awake behaving rats (Sprague Dawley) was used to determine the neurochemical response to local perfusions of METH and/or the succinate dehydrogenase inhibitor, malonate. METH (100 μM) in Dulbecco's saline medium was reverse-dialysed into one side of the striatum for 8 hours via a microdialysis probe, while the contralateral side was perfused with vehicle only. Extracellular concentrations of DA and glutamate were monitored simultaneously throughout the perfusion by HPLC/EC. In other experiments, malonate (50 mM, 100 mM or 200 mM), alone or in combination with METH (100 μM), was perfused on one side of the striatum while the other side was perfused with normal Dulbecco's. Seven days following drug treatment, tissue immediately adjacent to the probe site was dissected out and assayed for DA and 5-HT content by HPLC/EC.

Results and Discussion

The continuous local perfusion of METH alone increased DA release by 30 fold, similar to that seen following systemic administration, but did not increase extracellular glutamate and did not deplete DA or 5-HT content. Malonate perfusion increased both DA and glutamate overflow, and dose dependently decreased DA content (Table I). 5-HT content (Table II) was not as affected by malonate perfusions (200 mM malonate depleted DA by 60% and 5-HT by only 30%). When METH was co-perfused with 200 mM malonate, striatal DA content was reduced by 75% and to a greater extent than on the side perfused with malonate alone. Co-perfusion of METH and 200 mM malonate did not enhance 5-HT loss.

8th International Conference on In Vivo Methods, Stony Brook, NY, 1999.

	Perfused Alone	Perfused with 50 mM Malonate	Perfused with 100 mM Malonate	Perfused with 200 mM Malonate
Dulbecco's	114 ± 6.5	110 ± 11.4	69 ± 14.2	46 ± 6.0
METH	128 ± 14.3	112 ± 8.4	58 ± 6.0	31 ± 4.3

Table I. *Striatal DA Content (μg/pg protein) 7 days following drug treatment.*

	Perfused Alone	Perfused with 100 mM Malonate	Perfused with 200 mM Malonate
Dulbecco's	4.1 ± 0.3	4.5 ± 0.9	2.9 ± 0.4
METH	6.1 ± 1.0	4.5 ± 0.5	2.3 ± 0.3

Table II. *Striatal 5-HT Content (μg/pg protein) 7 days following drug treatment.*

Malonate alone damages DA systems while leaving 5-HT content relatively unaffected, suggesting that nerve terminals containing these neurotransmitters are differentially sensitive to the effects of mitochondrial inhibition. The failure of the local perfusion of METH to produce a long-term depletion of DA may be due to its lack of effect on glutamate release. Thus, increased extracellular DA concentrations are not sufficient to mediate METH-induced damage to DA and 5-HT terminals. Overall, the direct local perfusion of METH alone is not toxic to striatal DA terminals but synergizes with the local inhibition of energy metabolism to deplete DA content. Moreover, the inhibition of energy metabolism and the synergy with METH is selectively toxic to DA but not 5-HT neurons.

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Society for Neuroscience Annual Meeting, New Orleans, LA, 2000

CHRONIC UNPREDICTABLE STRESS AND METHAMPHETAMINE TOXICITY: ROLE OF 5HT-MEDIATED HYPERTHERMIA. L. Matuszewich* and B.K. Yamamoto. Department of Psychiatry, Case Western Reserve University, Cleveland, OH 44106.

Human and experimental animal studies have demonstrated that repeated exposure to stressful stimuli damages neurons or increases the probability of their damage by future insults. The serotonergic system may mediate the neurochemical vulnerability induced by chronic stress. Male rats exposed to 10 days of unpredictable stress had an increased mortality rate (71%) compared to non-stressed controls (0%) following injections of methamphetamine (METH) at neurotoxic doses (10 mg/kg x 4, every 2 hours). Stressed rats also had greater hyperthermic responses and more dopamine release in striatum compared to non-stressed controls after METH at the high dose (10 mg/kg x 4) and at a lower dose (7.5 mg/kg x 4)($p < .05$). Furthermore, one week following the lower dose of METH, stressed rats showed greater depletions in striatal dopamine tissue content ($p < .05$). To determine whether the potentiated hyperthermic response to METH in chronically stress rats is mediated by 5-$HT_{2A/C}$ receptors, either the 5-$HT_{2A/C}$ agonist (±)-DOI (1.5 mg/kg, i.p.) or saline was injected and body temperature measured every 15 minutes before and for 2 hours following DOI. DOI significantly increased body temperature in stressed and non-stressed rats compared to saline injected rats ($p < .05$). The DOI-induced increase in body temperature was potentiated in chronically stressed rats compared to non-stressed controls 75 and 90 minutes after the injection. This study supports our previous findings of a hyper-responsiveness of chronically stressed rats to METH and suggests that it is mediated by an alteration in 5-$HT_{2A/C}$ receptor function. Supported by DA05937-02, DA07606 and DAMD 17-99-1-9479.

Neurotoxic Treatment with Methamphetamine or MDMA Affect BDNF in the Frontal Cortex and Hippocampus.

Leslie Matuszewich & Bryan K. Yamamoto
Case Western Reserve University
Program in Basic & Clinical Neuroscience

The neurotrophin brain-derived neurotrophic factor (BDNF) has been shown to enhance the survival of neurons in the adult rat nervous system. Infusing BDNF into the neocortex during treatment with the serotonin neurotoxin p-chloroamphetamine increased the survival of serotonergic neurons in vivo (Mamounas et al., 1995). Similarly, administering BDNF to dopamine neurons in vitro decreased the susceptibility to the dopamine neurotoxin 6-hydroxy-dopamine (Spina et al., 1992). The psychostimulants 3,4-methylenedioxymethamphetamine (MDMA) and methamphetamine (METH) also act as neurotoxins to serotonin and dopamine neurons. The current study investigated the effect of MDMA or METH on BDNF concentrations in several brain regions. Male rats were treated with MDMA or METH (10 mg/kg, i.p.), or an equivalent volume of saline every 2 hours for a total of 4 injections. Twenty-four hours or 7 days after the 1st injection, the animals were sacrificed and the following brain regions were dissected: hippocampus, frontal cortex, striatum, and substantia nigra. Naïve controls were sacrificed immediately upon arrival into the laboratory, without systemic injections to measure basal levels of BDNF. To verify the toxicity of the systemic drug treatments, dopamine and serotonin tissue content were measured in tissue samples from one hemisphere in animals sacrificed 7 days after treatment. Treatment with METH, but not MDMA, resulted in depletions of dopamine tissue content in the striatum (65% depletion compared to saline injected controls). Frontal cortex and hippocampal tissue showed depletions in serotonin, but not dopamine, tissue content following treatment with either amphetamine (50% depletion of the cortex and 60% depletion of the hippocampus compared to saline injected controls). Once depletions were verified in subjects sacrificed 7 days after treatment, tissue from the contralateral hemisphere was used to measure BDNF concentrations. The tissue samples were homogenized in a lysis buffer and BDNF concentrations were determined with an ELISA (Promega BNDF Emax). As demonstrated in previous studies, the hippocampus (1.277 ng/mg protein) showed the highest concentration of BDNF compared to the frontal cortex (0.563 ng/mg protein) and the striatum (0.49 ng/mg protein). BDNF concentrations were decreased by 30% in the frontal cortex 24 hrs and 7 days following MDMA treatment, while treatment with METH resulted in decreased cortical BDNF 7 days after treatment only. BDNF concentrations in the hippocampus were increased 7 days after treatment with METH and MDMA by 187% and 162%, respectively. The localization of the effects of MDMA and METH in the hippocampus and frontal cortex suggests that the relationship is associated with the serotonergic system. Current studies are investigating the ability of ritaserin, a serotonin 2A/2C antagonist, co-injected with METH or MDMA to attenuate the effects of the amphetamines on BDNF.

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***Neurotoxicity of Amphetamines and Related Stimulants*,**
Washington D.C., 1999

Long-term effects of MDMA on stress-induced glutamate release

L. Matuszewich, M. E. Filon and B.K. Yamamoto
Program in Basic and Clinical Neuroscience
Department of Psychiatry, Case Western Reserve University, Cleveland. OH 44106

Introduction

Repeated exposure to environmental stress or to psychostimulant drugs, including (±)3,4-methylenedioxymethampetamine (MDMA), can damage neurons in the hippocampus. The neuronal damage can be blocked by impairing the secretion of corticosterone,[1,2] suggesting that the long-term toxicity induced by either stress or MDMA may be due to similar mechanisms. High doses of MDMA are selectively neurotoxic to serotonin (5-HT) neurons in several brain regions, including the hippocampus and the cortex[3]. Interactions between the central serotonergic system and the hypothalamic-pituitary axis (HPA) have been proposed,[4] with 5-HT contributing to the stress response. Treatment with the 5-HT uptake enhancer, tianeptine, prevented the stress- and corticosterone-induced atrophy of CA3 hippocampal neurons.[5] Thus, serotonin may contribute to the central effects of stress.

Serotonin may modulate the effects of stress by its interactions with the excitatory amino acid, glutamate. During acute stress, glutamate has been shown to increase in the hippocampus and cortex,[6,7] regions damaged by a neurotoxic dose of MDMA. Serotonin depletion with 5,7-dihydroxytryptamine decreased glutamate binding in the hippocampus[8]. Whether the serotonin system modulates stress-induced glutamate activity *in vivo* is unknown. Furthermore, the functional consequences of MDMA-induced 5-HT depletion in physiological responses to behavioral challenges, such as restraint stress, have not been clarified. The current experiment will investigate the effect of MDMA-induced serotonin depletion on glutamate efflux in the hippocampus during restraint stress using *in vivo* microdialysis.

Materials and Methods

In vivo microdialysis in awake behaving rats was used to measure extracellular glutamate efflux prior to, during and after restraint stress[6] in saline- and MDMA-pretreated rats. Male Sprague-Dawley rats (175-225 g) were treated with MDMA (10 mg/kg, i.p.) or an equivalent volume of saline every 2 hours for a total of 4 injections. Four days after treatment, rats were implanted with a guide cannula aimed above the dorsal hippocampus (A/P 3.2, M/L 2.0 from bregma). Dialysis probes as described previously[9] were inserted through the guide cannula into the hippocampus 24 hours prior to testing, 6 days after systemic treatment. On the day of microdialysis, Dulbecco's aCSF was perfused through the probes at 1.5 µl/min for 4 hours. Twenty minute samples were collected during each of the following conditions: 1 hour baseline period, 1 hour of restraint stress, and 1 hour after restraint stress, freely moving. Glutamate levels in the dialysate were measured by high performance liquid chromatography with electrochemical detection (HPLC-EC)[10]. Hippocampal tissue content levels of serotonin were measured with HPLC-ED to determine the level of serotonin depletion following MDMA treatment.

Results and Discussion

Glutamate efflux in the hippocampus increased 23-25% during the restraint stress sampling period in rats previously injected with MDMA, whereas in saline treated rats, glutamate increased 53-104%. Thus, the MDMA treated rats showed an attenuated glutamate response to restraint stress compared to saline controls ($p<0.05$). Following restraint stress, glutamate continued to increase gradually in MDMA treated rats, reaching significance by the final dialysate sample ($p<0.05$).

8th International Conference on In Vivo Methods, Stony Brook, NY, 1999.

Glutamate declined following stress in the saline treated rats, returning to baseline levels by the final sample. Serotonin tissue content in the hippocampus was depleted by 56% in MDMA treated rats when examined 7 days after drug administration.

Stress-induced glutamate increases in the hippocampus can be altered by partially depleting serotonin with a neurotoxic treatment of MDMA. Previous studies have demonstrated an increase in glutamate efflux in the hippocampus during restraint stress[6,7] as demonstrated in the saline treated rats in the current experiment. Central serotonergic systems are thought to modulate stress activation of the hypothalamic-pituitary axis. By depleting serotonin stores in terminal regions with the neurotoxin MDMA, the ability of the serotonergic system to activate central systems that respond to stress may be compromised. Serotonin release in the ventral hippocampus was shown to increase during inescapable shock[11]. It may be speculated that 5-HT release during stress modulates hippocampal glutamatergic activity, and that this serotonergic modulation of glutamate is disrupted by prior exposure to MDMA.

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EFFECTS OF CHRONIC UNPREDICTABLE STRESS ON MDMA AND METHAMPHETAMINE NEUROTOXICITY. L. Matuszewich[2]*, G.A. Gudelsky[1] and B.K. Yamamoto[2]. [1]College of Pharmacy, University of Cincinnati; [2]Program in Basic & Clinical Neuroscience, Department of Psychiatry, Case Western Reserve University, Cleveland, OH 44106.

Human and experimental animal studies have demonstrated that repeated exposure to stressful stimuli may damage neurons or increase the probability of their damage by future neurological insults. One reported consequence of exposure to stressful stimuli is a decrease in the growth factor, brain-derived neurotrophic factor (BDNF) mRNA in the hippocampus (Smith et al., 1995). BDNF has been suggested to be neurotrophic to both serotonin and dopamine neurons during various neurological insults. Whether stress potentiates the neurotoxic effects of the psychostimulants methamphetamine (METH) or (±)3,4-methylenedioxymethampetamine (MDMA) is unknown. The current study investigated the effects of chronic stress on: 1) BDNF protein expression; and 2) the neurotoxicity of METH and MDMA. Male rats were either exposed to daily stressors for 10 days or were weighed daily, but not exposed to other stressors. On the 11th day, rats were treated with a neurotoxic dosing regimen of METH or MDMA (10 mg/kg, i.p. for 4 injections) or an equivalent volume of saline. Twenty-four hours after the first injection, rats were killed and the striatum, hippocampus and frontal cortex were dissected. BDNF concentrations were determined with an ELISA (Promega BDNF Emax). BDNF levels were significantly increased in the striatum and frontal cortex of MDMA-treated, chronically stressed rats ($p < .05$). In contrast, BDNF levels in the hippocampus were significantly decreased in MDMA-treated, chronically stressed rats ($p < .05$). There were no significant effects of stress alone on BDNF protein levels in any brain region. Thus, exposure to chronic, unpredictable stress in conjunction with MDMA alters BDNF expression, which may contribute to the long-term neurotoxic effects of MDMA.

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LONG-TERM EFFECTS OF MDMA ON STRESS-INDUCED TRANSMITTER RELEASE. M.E. Filon[2], L. Matuszewich[2], G. A. Gudelsky[1] and B.K. Yamamoto[2]*. [1]College of Pharmacy, University of Cincinnati; [2]Program in Basic & Clinical Neuroscience, Department of Psychiatry, Case Western Reserve University, Cleveland, OH 44106.

High doses of (+)3,4-methylenedioxymethamphetamine (MDMA) are selectively neurotoxic to serotonin (5-HT) neurons in several brain regions, including the hippocampus and cortex. Although the 5-HT system has been implicated in the regulation of many behaviors, few studies have reported the long-term functional consequences of MDMA-induced 5-HT depletion. The current study investigated the effects of MDMA-induced 5-HT depletion on the release of neurotransmitters in response to acute, restraint stress. Male rats were treated with a neurotoxic dosing regimen of MDMA (10 mg/kg, i.p. for 4 injections) or an equivalent volume of saline. Seven days after treatment, in vivo microdialysis was used to assess extracellular dopamine, serotonin and glutamate in the dorsal hippocampus and prefrontal cortex during 1 hour of restraint stress. In saline treated control rats, there was a significant increase of glutamate (112%) and 5-HT (104%) over baseline levels in the hippocampus during restraint stress ($p < .05$). In the prefrontal cortex, similar increases in dopamine (46%) and 5-HT (79%) were observed in controls during restraint stress. Rats pretreated with MDMA, however, showed attenuated transmitter responses in the hippocampuus (glutamate--38%; 5-HT--27% of saline treated rats) and in the prefrontal cortex (dopamine--20%; 5-HT--10% of saline treated rats). Thus, the MDMA treated rats showed attenuated transmitter release in the hippocampus and prefrontal cortex in response to restraint stress compared to saline controls. The depletion of 5-HT stores in terminal regions with the neurotoxin MDMA may compromise the ability of the serotonergic system to activate central systems that respond to stress stimuli.

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